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Award Number: DAMD17-00-1-0497

TITLE: Endothelial Cell Morphogenesis and Angiogenesis Mediated by Carbohydrate-Binding Protein Galectin-3

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REPORT DATE: May 2003

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	May 2003	Final (15 Apr 00-15 Apr 03)

4. TITLE AND SUBTITLE
Endothelial Cell Morphogenesis and Angiogenesis Mediated by Carbohydrate-Binding Protein Galectin-3

5. FUNDING NUMBERS
DAMD17-00-1-0497

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Original contains color plates. All DTIC reproductions will be in black and white.

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)
Based on our preliminary findings involving the recombinant galectin-3 and low, high and null galectin-3 expressing breast cancer cells, we hypothesized that galectin-3 is an angiogenic stimulator and plays an important role in the progression of breast carcinoma as an alternate signaling pathway for the carbohydrate recognition. The purpose of the present study is to determine if a) galectin-3 has a role in breast cancer progression and angiogenesis and b) if MCP is able to inhibit progression/metastasis and angiogenesis in breast cancer. Our results indicate that galectin-3 redistributed from luminal to basal epithelial cells during breast cancer progression and might play a role in the invasiveness of certain cancer cells by interacting with stromal cells. Using a novel 3-dimensional in vitro co-culture system that permits growth and functional organization/differentiation of preneoplastic human breast epithelial cells and endothelial cells , we demonstrated that galectin-3 is an important mediator of epithelial-endothelial cell-cell interactions. Blocking galectin-3 expression by anti-sense transfection of galectin-3 in MDA-MB-435 cells resulted in loss of tumorigenic potential and transformed phenotype. Modified Citrus Pectin (MCP) is able to reduce tumor size, microvessel density as well as metastatic potential of MDA-MB-435 cells injected in nude mice. Administration of MCP at a later time i.e. after removal of primary tumor did not inhibit metastasis.

14. SUBJECT TERMS
Cancer biology, angiogenesis, prevention

15. NUMBER OF PAGES
54

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Final report

Introduction

Galectin-3 is a member of a growing family of carbohydrate-binding proteins sharing a conserved sequence of the sugar binding motif and an affinity for galactoside containing glycoconjugates (1). Galectin-3 is expressed in a wide range of neoplasms and is involved in multiple biological processes, including cell growth, adhesion, differentiation, inflammation, apoptosis, and metastasis through interactions with specific ligands (2-5). In human tumors, a direct relationship between galectin-3 levels and the stage of tumor progression has been demonstrated in a wide range of carcinomas (2, 6). There are some conflicting reports also, in which the level of galectin-3 has been reported to down-regulate with advanced grade cancer (7-9)

Previously it was suggested that the role of tumor cell galectin-3 in metastasis is to mediate cell-cell interaction leading to the formation of tumor cell emboli, which get arrested in the capillary bed of target organ (10). Based on this notion the carbohydrate-binding domain was utilized as a target for inhibition of experimental metastasis. Pectin, a complex highly branched polysaccharide rich in galactoside residues is present in cell walls of all plant tissues. In its native form it is unable to interact with galectin-3. But when citrus pectin (CP) is pH and temperature modified into MCP, which is a smaller, linear complex polysaccharide it is capable of combining with the carbohydrate-binding domain of galectin-3 (11). It was shown that when mouse melanoma cell line B16F1 was injected into mice with MCP, there was a dramatic reduction in their lung colonization properties (11). MCP was shown to inhibit galectin-3 induced functions, which involve its carbohydrate binding property.

The purpose of the present study is to determine if a) galectin-3 has a role in breast cancer progression and angiogenesis and b) if MCP is able to inhibit the progression / metastasis and angiogenesis in breast cancer. Results from the proposed studies will not only aid in understanding the role of galectin-3 in breast cancer progression, but also provide a way to evaluate the role of natural nontoxic competitive polysaccharide in prevention (preneoplasia) or treatment (metastasis) of breast cancer.

Body

In the last three years various experiments have been performed pertaining to the three aims that were proposed.

Aim 1: To establish the significance of galectin-3 expression and secretion in the progression and angiogenesis of human breast carcinoma:

To understand this aim, the following experiments were performed.

A: In situ hybridization and immunohistochemical Analysis of human breast cancer tissue:
The breast cancer sections were obtained from the Human Tissue Resource Core of the Karmanos Cancer Institute. Sections were stained with anti Factor VIII as a marker for blood vessel formation, and poly and monoclonal anti galectin-3 antibodies TIB166 and hL-31 respectively.

B: In situ hybridization and immunohistochemical analysis of MCF10AT1 and MCF10AT1 DCIS.com xenografts:
To get a more detailed information on relationship of galectin-3 expression and breast cancer progression, we also studied MCF10AT1, and MCF10DCIS.com xenografts. MCF10AT1 cells are T24 *Ha-ras*-transformed cells derived from normal behaving MCF10A human breast epithelial cells and form persistent premalignant lesions in immune deficient mice, whereas MCF10A cells do not grow in nude mice (12). Lesions formed by MCF10AT1 cells are composed of a heterogeneous spectrum of ductular tissues with a range of morphology that includes mild to moderate hyperplasia, atypical hyperplasia, ductal carcinoma *in situ* (DCIS), moderately differentiated carcinoma, undifferentiated carcinoma as well as histologically normal ducts (13, 14). The MCF10DCIS.com cell line (referred as DCIS.com) was cloned from a cell culture derived from a MCF10AT xenograft following two successive trochar passages (15). Injection of DCIS.com cells into nude mice results in lesions that are predominantly of the comedo-DCIS subtype (15).

Results: *In situ* hybridization analysis and immunohistochemical studies indicate that intense signals for galectin-3 mRNAs were localized in luminal epithelial cells in normal and hyperplastic ducts of premalignant MCF10AT1 xenografts and in normal and hyperplastic areas of cancerous tissues. However, whereas only trace or negligible galectin-3 mRNA signals were detected in early comedo-DCIS, i.e., prior to formation of central comedo-necrotic core, advanced DCIS lesions with conspicuous central comedo necrosis exhibited moderate galectin-3 mRNA signals that

were dispersed away from the comedo core, towards the periphery. Moderate to strong galectin-3 mRNA signals were also observed in fibroblasts of the extracellular matrix in and around the infiltrating cancer cells in human breast tissues.

The immunohistochemistry patterns of galectin-3 protein staining in normal, hyperplastic, comedo-DCIS and invasive breast tumors were similar to corresponding *in situ* hybridization patterns. Normal and hyperplastic ducts from human breast tissues and premalignant MCF10AT1 xenografts showed strong galectin-3 immunoreactivity in lumen and luminal epithelial cells. Early grades of DCIS lesions (day 20), i.e., prior to development of comedo necrosis, showed minimal and less prevalent galectin-3 immunoreactivity. In contrast, DCIS lesions (mid-grade: harvested at 40 days) and advanced DCIS lesions (harvested at 60 days) with manifested central comedo necrosis displayed intense to focally intense galectin-3 immunoreactivity in cells situated at the periphery or proximal to the stromal microenvironment. Consistent with galectin-3 protein distribution in comedo-DCIS, a high-risk precursor subtype for progression, invasive breast carcinomas displayed strong galectin-3 immunoreactivity in the extracellular space around infiltrating tumor cells. These data reveal a transitional shift in galectin-3 expression and distribution with breast cancer progression and suggest acquisition and/or assumption of novel galectin-3 mediated functional interactions with the stromal microenvironment that are probably necessary for progression.

These results have been summarized in a manuscript that is being submitted to American Journal of Pathology (Appendix 1).

C: Anti-Sense Transfection of galectin-3 in metastatic breast cancer cell line MDA-MB-435:

Galectin-3 was transfected in MDA-MB-435 cells in antisense orientation and their tumorigenic and metastatic properties were studied. Our results indicate that expression of galectin-3 is important for the maintenance of the transformed and tumorigenic phenotype of MDA-MB-435 breast carcinoma cells. The clones in which the galectin-3 expression was blocked showed an altered morphology, contact inhibition, a reduced growth in soft agar, and a reduced growth rate in serum free conditions. When these clones were injected in the mammary fat pad region of nude mice, there was no tumor formation with $1x10^6$ cells, whereas when $5x10^6$ cells were injected, there was a 30% tumorigenesis compared to the 100% in non-transfected controls. The results have been compiled in the form of a publication in Clinical Cancer Research (Appendix 2)

Sense transfection of galectin-3 in SK-Br-3 cells was not successful.

D: Galectin-3 expression in breast cancer cell lines in relation to their angiogenic properties in nude mice:

The expression and secretion of galectin-3 in breast cancer cell lines were studied by Western blot analysis (Fig1).

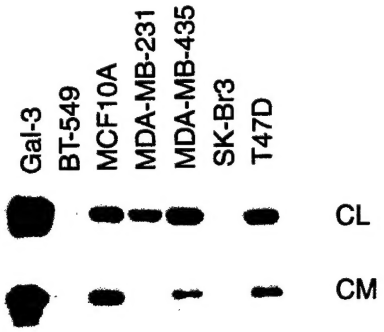

Fig 1: Western blot analysis of galectin-3 expression in breast cancer cell lines. Galectin-3 is expressed in MCF10A, MDA-MB-231, MDA-MB-435 and T47D cells, and is not expressed in BT-549 and SK-Br-3 cells, whereas the secretion of galectin-3 is seen only in T47D, MCF10A and MDA-MB-435 cells.

The angiogenic potential was determined by Matrigel plug assay (Fig.2).

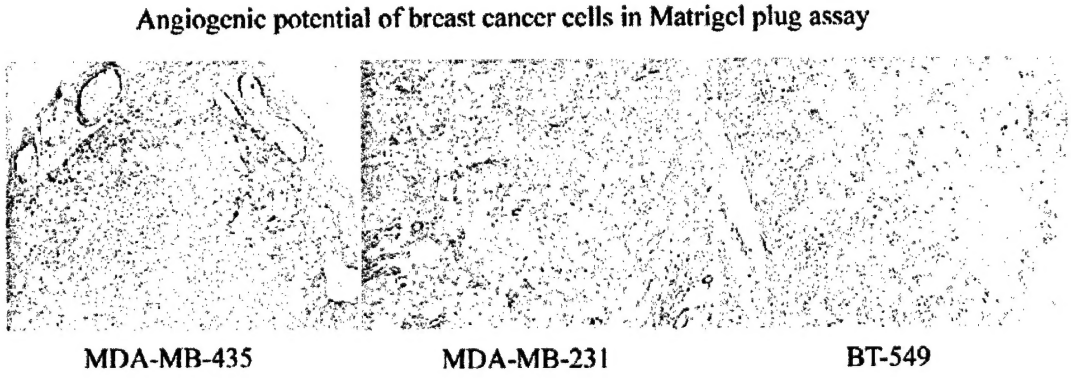

Fig. 2: 1x106 cells were mixed with Matrigel and injected in the dorso-lateral region of nude mice subcutaneously. We selected three representative cell lines, one null galectin-3 expressing (BT-549), one galectin-3 expressing, but not secreting (MDA-MB-231), one that secretes galectin-3 and shows high expression (MDA-MB-435). After one

week, the plugs were removed, embedded in paraffin, sectioned and stained for blood vessel formation with smooth muscle actin.

No blood vessels were observed in BT-549 plugs, large blood vessels were observed in MDA-MB-435 plugs and small vessels with incomplete lumens were seen in MDA-MB-231 plugs. The results indicate a direct correlation between galectin-3 expression, secretion and angiogenic potential of breast cancer cell lines.

Aim 2: To determine whether gal-3 influences preneoplastic conversion of MCF10A cells by switching from antiangiogenic to angiogenic phenotype.

We have shown earlier that galectin-3 is involved with in vitro and in vivo angiogenesis. Previous studies from our laboratory have shown differences in the ability of normal MCF10A versus preneoplastic MCF10AT-EIII8 cells to interact and establish stable functional interactions with endothelial cells (13, 16, 17), which is a part of angiogenic phenomenon. Since our initial preliminary analysis showed lower levels of gal-3 in MCF10A cells as compared to preneoplastic MCF10AT-EIII8 cells, we speculated that perhaps the differences in gal-3 expression levels contributed to differences in *in vitro* behavior. However, subsequent repetition of Western blot analysis of gal-3 levels revealed no significant difference in steady-state levels of gal-3 in cell lysates or conditioned media (secreted) between MCF10A and MCF10AT-EIII8 cells. Thus, the experiments proposed in the grant proposal to study the effects of stable expression of sense and antisense gal-3 in MCF10A and MCF10AT- EIII8 cells are not warranted. Instead, we have characterized expression of gal-3 mRNA and protein and examined its potential role in mediating heterotypic epithelial-endothelial cell interaction by using a novel three-dimensional *in vitro* co-culture model.

Our results on the role of galectin-3 in epithelial endothelial cell-cell interactions can be summarized as follows:

a) Coculture of epithelial cells with endothelial cells enhances galectin-3 secretion and specific proteolytic cleavage of secreted galectin-3, which seems to be regulated by MCP and CP
b) Galectin-3 is required for maintenance of stable breast epithelial endothelial heterotypic interactions
c) Breast epithelium is the major source of galectin-3

The results have been summarized as part of the manuscript (Appendix1).

Aim 3: To study the effects of MCP and galectin-3 specific antibodies on angiogenesis and metastasis of breast cancer cells.

This aim consists of two parts.

A: Effect of MCP on galectin-3 induced angiogenesis and metastasis:
To evaluate the effect of MCP on tumor induced angiogenesis, we proposed to feed the mice with 0.1%, 0.5% and 1% solution of MCP in water and inject MDA-MB-435 cells in the mammary fat pad of nude mice. We planned to follow three different feeding regimens with MCP i) The mice will be started on MCP water 2 weeks prior to the injections, ii) MCP water will be started at the same time as the injections, iii) MCP water will be started after the appearance of primary tumor. In all the treatments MCP water administration will be continued until completion of the experiment. Control mice will be fed on regular water.
Before starting the complete experiment, a trial experiment was performed. In this experiment 10 mice were started on 1% MCP two weeks prior to the injections, and at the time of removal of the primary tumor. The mice were sacrificed after 16 weeks and lung metastasis was observed.
Results: There was no metastasis in MCP fed mice, but the mice that were transferred to MCP feeding after removal of the primary tumor showed no inhibition in metastasis. The experiment needs to be repeated with the other feeding regimens.
In the second experiment, we started the mice on MCP (1.0%) solutions a week prior to injections with $0.5x10^6$ MDA-MB-435 cells in the mammary fat pad region. The primary tumor was removed at 7-8 weeks and the mice were sacrificed after 8 more weeks.
We have shown that there is a significant reduction in tumor growth and metastasis in MCP fed mice. The microvessel density was measured after 4 weeks and the counts showed that the mice fed on water had almost three times more vessels than the MCP fed group. We evaluated these results in relation to the ability of MCP to interfere with the binding of galectin-3 to its receptors on endothelial cells leading to an inhibition of endothelial cell morphogenesis and angiogenesis. Our results show that MCP inhibits the binding of recombinant galectin-3 to HUVEC, as well as of MDA-MB-435 cells. It inhibits the chemotaxis of HUVEC towards galectin-3 in a dose dependent manner. It also inhibits the capillary tube formation by HUVEC on Matrigel. The data on the *in vitro* and *in vivo* inhibition of angiogenesis and metastasis by MCP is presented in a manuscript submitted to JNCI (Appendix 2).

Aim 3b: Matrigel plug assays in the presence of MCP:

Breast cancer cells ($1x10^6$) were mixed with 300 ul Matrigel and injected in nude mice subcutaneously dorsolaterally. After one week the plugs

were removed, embedded in paraffin, sectioned and stained for the presence of blood vessels. These experiments have been performed. Matrigel plugs have been embedded and sectioned and will be stained for CD31 and Factor VIII antibodies. We have only analyzed the results from MDA-MB-231 cells, which show a significant reduction in the number of blood vessels (Fig.3).

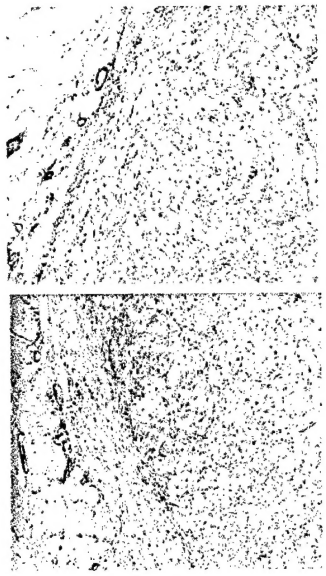

Fig.3: Effect of MCP on blood vessel formation in Matrigel plug assays utilizing MDA-MB-435 cells. Top panel is the Matrigel plug with MDA-MB-231 cells alone, and bottom panel is MDA-MB-231 cells mixed with modified citrus pectin. The blood vessels are seen only on the periphery in MCP treated plugs.

Key Research Accomplishments:

We have shown that galectin-3 is important for tumorigenic phenotype and metastatic potential of breast cancer cells MDA-MB-435. Blocking galectin-3 expression in this metastatic and tumorigenic cell line led to altered morphology, loss of serum independent growth, acquisition of growth inhibition properties by cell contact, and abrogation of anchorage independent growth.

During breast cancer progression, normal and hyperplastic ducts express elevated levels of galectin-3 mRNA and protein in the luminal epithelial cells, and is down-regulated in early grades of DCIS. Interestingly, galectin-3 is re-expressed in the peripheral tumor cells as DCIS lesions evolve and/or progress to comedo-DCIS. This pattern of galectin-3 mRNA and protein expression in the peripheral tumor cells is maintained in invasive breast carcinomas. These data suggest that galectin-3 expression is not only associated with specific morphologic precursor-subtypes of breast cancer but is also accompanied by a transitional switch in expression from luminal to basal epithelial cells that is coincident with acquisition of invasive potential. Such localized expression of galectin-3 in cancer cells proximal to the stroma could lead to increase in invasive potential by inducing better interactions with the stromal counterparts.

Using a novel three dimensional *in vitro* culture system that facilitates functional organization and differentiation of preneoplastic epithelial and endothelial cells, we have shown that blocking galectin-3 activity by galectin-3 neutralizing antibodies causes inhibition of epithelial-endothelial cell-interaction and growth. In situ hybridization and immunocytochemistry analysis showed that the preneoplastic epithelial cells are the major source of galectin-3.

Modified Citrus Pectin (MCP) inhibited binding of galectin-3 and galectin-3 expressing cells MDA-MB-435 to human umbilical vein endothelial cells (HUVEC) and blocked chemotaxis and in vitro morphogenesis of HUVEC in a dose dependent manner. Tumor growth, angiogenesis and spontaneous metastasis were significantly inhibited by MCP.

Reportable Outcomes:
This grant proposal has resulted in the following publications:

1.Honjo, Y., Nangia-Makker, P., Inohara, H., Raz, A. Down regulation of galectin-3 suppresses tumorigenicity of human breast carcinoma cells. Clin. Cancer Res. 7(3):661- 668,2001.

2. Nangia-Makker, P., Honjo, Y., Hogan, V., Baccarini, S., Raz, A. (2002) Inhibition of human cancer growth and metastasis by oral intake of modified citrus pectin. J. Natl. Cancer Inst. 94 (24) 1854-1862

3. Nangia-Makker, P., Baccarini, S., and Raz, A. Carbohydrate-recognition and angiogenesis. In: Cancer and Metastasis Reviews. 19:51-57, 2000

4. Nangia-Makker, P., Baccarini, S., Raz, A. Carbohydrate-binding protein galectin-3: its role in metastasis, angiogenesis and apoptosis. In: Research Trends: Current Topics in Biomedical Research. U.Ramachandran (ed) Vol 3, pp 229-236, 2000.

5. Nangia-Makker, P., Conklin, J., Hogan, V., Raz, A. Carbohydrate-binding proteins in cancer and their ligands as therapeutic agents. Trends in Molecular Medicine vol 8(4), 2002, pp187-192

6. Shekhar, P.V.M., Nangia-Makker, P., Tait, L., Miller, F., Raz, A. Alterations in galectin-3 expression and distribution correlate with breast cancer progression: Functional analysis of galectin-3 in breast epithelial-endothelial interactions (submitted)

Abstracts:

1. Nangia-Makker, P., Honjo, Y., Hogan, V., Raz, A. (2000) Inhibition of angiogenesis by a natural polysaccharide: MCP. 91st Annual meeting of the American association of Cancer Research.
2. Honjo, Y., Nangia-Makker, P., Inohara, H. Raz, A. (2000) Loss of transformed phenotype and tumorigenicity of human breast cancer cell line by inhibition of galectin-3 expression. 91st Annual meeting of the American association of Cancer Research.
3. Nangia-Makker, P., Hogan, V., Honjo, Y., Baccarini, L., Bresalier, R., Raz, A. (2002) A simple sugar- modified citrus pectin acts as a potential anti-metastatic non-toxic therapeutic agent. 93rd Annual Meeting of the American Association of Cancer Research
4. Nangia-Makker, P. Shekhar, PVM, Raz, A. (2002) Role of galectin-3 in breast cancer progression and its inhibition by a natural non-toxic

polysaccharide. Era of Hope, Department of Defense Breast Cancer Research Program Meeting, Orlando, Florida, Sept 25-28.

5. Nangia-Makker, P. (2002) Carbohydrate-binding protein galectin-3 and its ligands. New trends in Cancer Therapy. 3rd International Cancer Congress, Rovigo, Venice, Italy, Dec3-6, 2002.
6. Shekhar, PVM, Tait, L., Nangia-Makker, P., Raz, A. (2003) Role of galectin-3 in endothelial epithelial interactions and its distribution in breast cancer progression. 94th Annual Meeting of the American Association of Cancer Research.

Conclusions

Galectin-3 plays a significant role in the metastatic phenotype of many cancer types. However, in some epithelial cancers, there have been conflicting reports on the role of galectin-3 in cancer progression. Our data indicate that in breast cancer, the distribution of galectin-3 changes from luminal epithelial cells to basal cells, when the cancer progresses from early stage DCIS to late stage DCIS lesions, indicating that it might be involved with invasiveness of the cells, by interacting with the stroma. Our data indicate that galectin-3 is important for facilitating epithelial-endothelial cell interactions and for the maintenance of tumorigenic and metastatic phenotype of breast cancer cells MDA-MB-435 by stimulating endothelial cells morphogenesis and angiogenesis. Modified Citrus Pectin (MCP) inhibits the binding of galectin-3 to its endothelial receptors and inhibits angiogenesis and metastasis of breast cancer.

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Appendices:

1. Manuscript submitted
2. Manuscript in Clinical Cancer Research
3. Manuscript in J. Natl. Cancer Inst.

Alterations in galectin-3 expression and distribution correlate with breast cancer progression: Functional analysis of galectin-3 in breast epithelial-endothelial interactions

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Running Title: Galectin-3 and breast cancer progression

Key words: epithelial-endothelial interactions, *in vitro* angiogenesis, three-dimensional coculture, comedo-ductal carcinoma *in situ*, proteolytic processing

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Abstract

Galectin-3 is a member of a growing family of carbohydrate-binding proteins sharing a conserved sequence of the sugar-binding motif and an affinity for galactoside containing glyco-conjugates. A relationship between galectin-3 levels and the stage of tumor progression has been demonstrated in a wide range of carcinomas, whereas in some epithelial cancers the reports on galectin-3 expression and tumor progression were conflicting. To understand the mechanistic role of galectin-3 in breast cancer progression and angiogenesis we have used a novel three dimensional endothelial epithelial coculture model which permits growth and functional organization / differentiation of both preneoplastic human breast epithelial cells (MCF10A EIII8) and endothelial cells (HUVEC), and performed a detailed analysis of expression and distribution of galectin-3 mRNA and protein in human breast tissue and MCF10AT xenografts. Treatment of MCF10A EIII8/HUVEC co-cultures with anti-galectin-3 antibodies inhibited both homotypic and heterotypic interactions and organization in a dose dependent fashion whereas treatment with corresponding normal IgGs had no effect, indicating that galectin-3 might play an important role in endothelial epithelial cell interactions.

The immunohistochemical analysis of breast cancer tissues and MCF10AT xenografts showed high levels of galectin-3 mRNA and protein localized in luminal epithelial cells in normal and hyperplastic ducts, whereas only trace galectin-3 mRNA and protein signals were detected in early grades of comedo positive DCIS lesions. However, in advanced grades of comedo positive DCIS lesions, which confers high risk of progression, galectin-3 mRNA and protein signals reappeared and were predominantly localized to peripheral or basally situated tumor cells. Moderate to strong galectin-3 signals were observed in most of the fibroblasts and ECM in and around the tumor cells in invasive carcinomas.

These data suggest that galectin-3 expression is probably associated with a transitional switch in expression from luminal to basal epithelial cells that is consistent with acquisition of invasive potential. Such localized expression of galectin-3 in cancer cells proximal to stroma could lead to increased invasive potential by inducing better interactions with stromal cells.

Introduction

Galectins are a family of nonintegrin β-galactoside binding lectins with related amino acid sequences [1]. Galectin-3 formerly known as CBP35, Mac-2 and εBP, due to its affinity for IgE [1] and HLB31[2], due to its affinity for laminin is a 31 kDa-galactoside-binding lectin and a member of the galectin family. Galectin-3 is widely expressed and secreted by myeloid and epithelioid cells [3 4] and binds polylactosamine glycans [5]. The lectin is associated with the plasma membrane and is secreted in the extracellular space [6] where it mediates cell-cell and cell-matrix interactions through its ability to bind to a variety of lactosamine-containing glycoconjugates for review [7 8]. Galectin-3 is composed of an amino terminal half containing Gly-X-Tyr tandem repeats that are characteristic of collagens and a carboxyl terminal half containing the carbohydrate binding domain [1].

Although the precise role of galectin-3 remains to be determined, several studies including ours have shown that expression of galectin-3 is positively correlated with the metastatic potential of several tumorigenic cell lines Reviewed in [9]. However, the generality of these findings in relation to epithelial-cell derived human tumors is not clear as increases and decreases in galectin-3 have been reported during malignant progression of several cancers [9]. In human colorectal carcinoma, galectin-3 has been reported to increase [10] or decrease [11,12] with progression to the metastatic state. Similarly, expression of galectin-3 is downregulated in prostate [13], ovarian [14] and breast [11,15] cancers.

We have previously reported that galectin-3 can induce endothelial cell morphogenesis *in vitro* and angiogenesis *in vivo* [16]. Binding of galectin-3 to the endothelial cell surface is dependent on its carbohydrate domain as binding is specifically inhibited by the competitive dissacharide lactose or modified citrus pectin [16]. Here, in order to further characterize the role of galectin-3 in endothelial morphogenesis, we have utilized a novel three-dimensional coculture system of *in vitro* angiogenesis that permits reciprocal functional epithelial-endothelial cell-cell and cell-matrix interactions [17]. Our results show that galectin-3 is important for stabilization of epithelial-endothelial interaction networks as immunoneutalization with galectin-3 antibodies specifically abolishes these interactions. *In situ* hybridization and immunohistochemical analyses of galectin-3 mRNA and protein, respectively, showed that breast epithelium is the major source of galectin-3. Expression and distribution of galectin-3 mRNA and protein were also examined in cancerous breast tumors and in premalignant and comedo-DCIS xenografts to further evaluate influence of galectin-3 on breast tumor growth and progression. Our findings show a switch in expression and distribution of galectin-3 from the luminal epithelium towards the periphery. These data suggest that such a transitional shift in galectin-3 expression, which is coincident with breast cancer progression, may facilitate novel galectin-3-mediated stromal-epithelial interactions that are probably required for invasion and metastatic progression.

Materials and Methods

Cell lines and xenografts. These studies utilized the following cell lines: MCF10AT1, MCF10AT1-EIII8 and MCF10DCIS.com cells. MCF10AT1 cells are T24 *Ha-ras*-transformed cells derived from normal behaving MCF10A human breast epithelial cells

and form persistent premalignant lesions in immune deficient mice, whereas MCF10A cells do not grow in nude mice [18]. Lesions formed by MCF10AT1 cells are composed of a heterogeneous spectrum of ductular tissues with a range of morphology that includes mild to moderate hyperplasia, atypical hyperplasia, ductal carcinoma *in situ* (DCIS), moderately differentiated carcinoma, undifferentiated carcinoma as well as histologically normal ducts [19]. MCF10AT1-EIII8 (referred as EIII8) cells are premalignant epithelial cells that were derived from lesions of MCF10AT1 cells arising in 17 β–estradiol-supplemented animals and respond to estradiol with increased growth *in vitro* and *in vivo* [Shekhar, 1998 #177;Shekhar, 2000 #176; [Visscher, 2001 #171]. The MCF10DCIS.com cell line (referred as DCIS.com) was cloned from a cell culture derived from a MCF10AT xenograft following two successive trochar passages [20]. Injection of DCIS.com cells into nude mice results in lesions that are predominantly of the comedo-DCIS subtype [20].

MCF10AT1 and EIII8 cells were maintained in DMEM-F12 medium supplemented with 0.1 μg/ml cholera toxin, 0.02 μg/ml EGF, 10 μg/ml insulin, 0.5 μg/ml hydrocortisone, 100 U/ml penicillin, 100 μg/ml streptomycin and 5% horse serum (Shekhar et al, 2000). DCIS.com cells were maintained in DMEM-F12 medium supplemented with 5% horse serum [20]. MCF10AT1 and DCIS.com xenografts were generated by injecting $1X10^7$ MCF10AT1 [21] or DCIS.com [20] cells into nude mice and lesions harvested at 100 (MCF10AT1), 22, 40 or 61 (DCIS.com) days.

HUVECs (purchased from American Type Tissue Culture Collection) at passage 13 were maintained in Endothelial Serum Free Basal growth Medium (SFM, Life Technologies, Inc., Grand Island, NY) supplemented with 10 ng/ml EGF, 20 ng/ml bFGF, and 10 μg/ml fibronectin. Cultures were maintained in a humidified atmosphere containing 5% CO_2 in air.

Three-dimensional culture of EIII8-HUVEC cells (heterotypic) on reconstituted basement membrane. 50 x 10^3 EIII8 cells were mixed with an equal number of HUVEC cells and seeded onto chamber slides coated with reconstituted basement membrane (Matrigel) in SFM as described by Shekhar *et al* [22]. Twenty-four hours after seeding, 0.5 or 1 μg of rabbit polyclonal (HL-31) or rat monoclonal (TIB-166) anti-galectin-3 antibody was added. Control cultures were either untreated or treated with similar amounts of the corresponding normal rabbit or rat IgG. Effects of antibody or normal IgGs on EIII8-HUVEC interaction and growth were measured on day 5 by counting trypan blue-excluded cells from dispase-treated cultures [22].

Preparation of conditioned media. EIII8 cells (50 x 10^3) were seeded alone or mixed with an equivalent number of HUVEC cells on Matrigel in SFM medium as described before. Cells were incubated for 6 h to attach, and media were replaced with fresh SFM. Cultures were also treated with 0.01 or 0.1% of pH modified citrus pectin (MCP) or control citrus pectin (CP; Sigma Chemical Co., St. Louis, MO), prepared as described by Nangia-Makker *et al* [23]. Culture media from homotypic and heterotypic cultures were removed on day 5, centrifuged to remove debris and used for Western analysis. After removal of media, matrix containing the three-dimensional structures was either solubilized for SDS-PAGE and Western blot analysis or fixed in buffered formalin for evaluation of galectin-3 mRNA and protein.

Western blot analysis. Steady-state levels of intact and cleaved galectin-3 proteins secreted into conditioned media and those present in EIII8-HUVEC cell lysates of untreated, MCP- and CP-treated cultures at day 5 were determined by subjecting 20 or 50 μg total protein, respectively, to SDS-PAGE on 7-15% gradient polyacrylamide gels and Western blot analysis. HL-31 polyclonal antibody recognizes both the intact 31 kDa and cleaved galectin-3 proteins, whereas TIB-166 monoclonal antibody recognizes only the intact galectin-3 protein. Immunoreactive bands were visualized by chemiluminescence, and band intensities were quantitated with a Model 300A densitometer (Molecular Dynamics, Sunnyvale, CA).

Preparation of DIG-labeled galectin-3 probes. A 353 bp fragment of human galectin-3 was amplified by PCR using primers specific for human galectin-3 corresponding to nucleotides 382-734 (GenBank Accession # M57710) and subcloned into pCRII TA-cloning vector (Invitrogen). Plasmid DNAs were isolated from bacterial colonies and screened for orientation and sequence confirmation. Digoxigenin-labeled riboprobes specific for the sense or antisense strands of galectin-3 were synthesized from appropriately linearized plasmids using the DIG-RNA labeling kit (Roche) and the appropriate RNA polymerase (Promega Corp., Madison, WI) plus 10 U of recombinant RNase inhibitor. The specificity of each probe was verified by hybridization to originating plasmids and negative controls.

***In situ* Hybridization analysis.** Formalin-fixed paraffin embedded sections prepared from EIII8-HUVEC three dimensional cocultures, MCF10AT1- and MCF10DCIS.com-derived xenografts, and human breast tumors were deparaffinized and utilized for detection of galectin-3 mRNA and galectin-3 protein by *in situ* hybridization (ISH) and immunohistochemical analysis (see below), respectively. ISH was performed as previously reported using DIG-labeled cRNA probes [24,25]. Tissue sections were processed consecutively, one slide being incubated with the sense probe and other with the antisense probe. Four-μm thick tissue sections were deparaffinized, rehydrated and incubated in 0.2 N HCl for 20 min. Slides were rinsed with 2 X SSC, and tissue sections were permeabilized with proteinase K at a concentration of 5 μg/ml for 15 min at 37°C. Following fixation with 4% paraformaldehyde and washing in 2 X SSC, the sections were prehybrizided for 2 h at 42° C in buffer containing 5 X SSC/50% formamide/5 X Denhardt's reagent and 250 μg/ml yeast tRNA. Hybridization was performed overnight at the same temperature in 5 X SSC/50% formamide/5 X Denhardt's reagent containing 500 μg/ml yeast tRNA and 10% dextran sulfate. The final concentration of DIG-labeled cRNA galectin-3 probes (sense and antisense) was approximately 2 ng/μl. After hybridization, excess probe was removed by washing in 2 X SSC and treatment with RNase (100 U/ml RNase T1 and 0.2 μg/ml DNase-free RNase A) at 37°C for 15 min. Slides were washed at 42°C in 2 X SSC (15 min) and twice in 0.5 X SSC (15 min each). The sections were incubated with an anti-digoxigenin antibody conjugated with horse radish peroxidase and color developed using Vectastain ABC kit (Vector Labs).

Immunohistochemistry. Galectin-3 protein was localized on corresponding deparaffinized sections of EIII8-HUVEC cocultures, MCF10AT1- and

MCF10DCIS.com-derived lesions, and human breast tumor tissues by immunohistochemistry using monoclonal TIB-166 galectin-3 antibody. The identity and functionality of endothelial and epithelial cells in the EIII8-HUVEC three-dimensional cocultures were established by immunostaining the sections with cd31, factor VIII (endothelial-specific) or pan-cytokeratin (epithelial-specific) antibodies. Slides were overlaid with avidin-biotin conjugated goat anti-mouse or anti-rat IgG, incubated in peroxidase substrate solution (3,3'-diaminobenzidine; VectaStain ABC kit) and counterstained with hematoxylin.

Statistical analysis. Specific differences among treatments were examined using the Student's *t* test. Statistical significance was determined with $P<0.005$ considered as statistically significant.

RESULTS

Coculture with endothelial cells enhances galectin-3 secretion and specific proteolytic cleavage of secreted galectin-3. Regulation by MCP and CP. Galectin-3 protein levels and proteolytic processing in homotypic (EIII8) and heterotypic (EIII8-HUVEC) three-dimensional cultures treated with CP or MCP were determined by Western blot analysis of conditioned media and cell lysates. Immunoblot analysis of conditioned media with polyclonal HL-31 anti-galectin-3 antibody showed the presence of ~31 and 27 kDa bands, the latter indicative of proteolytic processing of galectin-3 (Fig. 1A). That the 27 kDa band represents proteolytic clipping of galectin-3 was confirmed by Western analysis with TIB-166 monoclonal antibody, which revealed only the intact 31 kDa galectin-3 protein (Fig. 1B). Since MCP has been demonstrated to inhibit binding of galectin-3 to HUVECs [23], we evaluated the effects of CP and MCP on secretion and processing of galectin-3 in EIII8-HUVEC cocultures. Consistent with previous data, results of Fig. 1A show a 50-70% decrease in levels of total galectin-3 secreted in EIII8-HUVEC cocultures treated with MCP as compared to those treated with CP or untreated cultures, respectively. However, it is interesting to note that no differences in proteolytic processing of secreted galectin-3 were observed between control or treated EIII8-HUVEC, or homotypic EIII8 cultures. Analysis of corresponding lysates with HL-31 antibodies showed only the presence of 31 kDa protein as the major product whereas the smaller 27 kDa band was undetectable (Fig. 1A'). Longer exposure of the blots revealed the presence of minor band at ~22 kDa in cultures treated with CP but were absent in MCP-treated cultures (Fig. 1A'). Immunoblotting of cell lysates with TIB-166 antibody confirmed the presence of the intact form in homotypic EIII8 and pectin-treated EIII8-HUVEC cultures (Fig. 1B'). These results suggest that galectin-3 is largely protected from proteolytic processing in the cells as opposed to the secreted protein that is highly sensitive to proteolytic cleavage at a site that is distinct from that of intracellular galectin-3.

Galectin-3 is required for maintenance of stable breast epithelial-endothelial heterotypic interactions. In order to further evaluate the functional role of galectin-3 in mediation of stable heterotypic interactions between premalignant breast epithelial cells and endothelial cells, we tested the effects of galectin-3 antibodies on EIII8-HUVEC

cocultures. Results from Fig. 2 show that whereas addition of the corresponding rat or rabbit normal IgGs failed to significantly influence growth and organization of EIII8-HUVEC cocultures as compared to control untreated cocultures, inclusion of equivalent amounts of polyclonal HL-31 or monoclonal TIB-166 anti-galectin-3 antibodies produced a dose-dependent inhibition of growth and loss of organization in EIII8-HUVEC cocultures. Addition of 0.5 or 1 μg of HL-31 anti-galectin-3 antibody caused ~2 or 3.8-fold decrease in growth ($P<0.001$), respectively, and complete loss of organization when compared to control untreated cultures. Similar addition of 0.5 or 1 μg of the monoclonal TIB166 anti-galectin-3 antibody resulted in 2 to 3-fold decrease in growth ($P<0.005$), respectively, and loss of organization as compared to control cultures. These data indicate that galectin-3 plays an important role in stabilization of heterotypic interactions between premalignant EIII8 and endothelial cells.

Breast epithelium is the major source of galectin-3. We have previously reported the presence of an intimate and symbiotic interaction between epithelial and endothelial cell populations that is mutually beneficial as observed by the colocalization of branching ductal-alveolar outgrowths with endothelial cell enriched regions (Shekhar et al, 2000). Immunocytochemical staining of formalin-fixed paraffin-embedded sections of EIII8-HUVEC cocultures with cd31, Factor VIII, pan-cytokeratin and PCNA antibodies revealed the presence of functional endothelial cells juxtaposed to a proliferative epithelium (Fig. 3). Immunostaining with TIB-166 galectin-3 antibody showed the presence of strong galectin-3 specific immunoreactivity exclusively in the epithelial compartment with discernibly higher reactivities in epithelial cells that are juxtaposed to endothelial cells (Fig. 3, panels G and H).

In situ hybridization analysis of galectin-3 mRNA signals in serial sections of EIII8-HUVEC cocultures showed the presence of galectin-3 mRNA signals at varying intensities throughout the proliferating epithelium but not in the endothelial compartment (Fig. 4A). The ISH signals are specific to galectin-3 mRNA expression as it was detected only with the antisense probe. Hybridization with the sense probe failed to produce an ISH signal and pretreatment of slides with RNase abolished the hybridization signals (Fig. 4B). These data suggest that galectin-3 protein regulating heterotypic EIII8-HUVEC interactions, growth and endothelial cell function is derived exclusively from galectin-3 messages transcribed in the epithelial compartment.

Alterations in galectin-3 expression and distribution in preneoplasia vs. comedo-DCIS vs. invasive breast carcinomas implicate potential differences in galectin-3 function during progression. *In situ* hybridization analysis was performed to determine the site and levels of galectin-3 mRNA expression in premalignant (MCF10AT1 xenografts), comedo-DCIS (DCIS.com xenografts), and breast carcinomas. Intense signals for galectin-3 mRNAs were localized in luminal epithelial cells in normal and hyperplastic ducts of premalignant MCF10AT1 xenografts (Fig. 5, panel A) and in normal and hyperplastic areas of cancerous tissues (Fig. 6, panels A and B). However, whereas only trace or negligible galectin-3 mRNA signals were detected in early comedo-DCIS, i.e., prior to formation of central comedo-necrotic core (Fig. 7, panel A), advanced DCIS lesions with conspicuous central comedo necrosis exhibited moderate galectin-3 mRNA signals that were dispersed away from the comedo core, towards the

periphery (Fig. 7, panels B,C). Moderate to strong galectin-3 mRNA signals were also observed in fibroblasts of the extracellular matrix in and around the infiltrating cancer cells in human breast tissues (Fig. 6, panel C).

The immunohistochemistry patterns of galectin-3 protein staining in normal, hyperplastic, comedo-DCIS and invasive breast tumors were similar to corresponding *in situ* hybridization patterns. Normal and hyperplastic ducts from human breast tissues and premalignant MCF10AT1 xenografts showed strong galectin-3 immunoreactivity in lumen and luminal epithelial cells (Fig. 5, panel A; Fig. 6, panels A" and B"). Early grades of DCIS lesions (day 20), i.e., prior to development of comedo necrosis, showed minimal and less prevalent galectin-3 immunoreactivity (Fig. 7, panel D). In contrast, DCIS lesions (mid-grade: harvested at 40 days) and advanced DCIS lesions (harvested at 60 days) with manifested central comedo necrosis displayed intense to focally intense galectin-3 immunoreactivity in cells situated at the periphery or proximal to the stromal microenvironment (Fig. 7, panels E and F). Consistent with galectin-3 protein distribution in comedo-DCIS, a high-risk precursor subtype for progression, invasive breast carcinomas displayed strong galectin-3 immunoreactivity in the extracellular space around infiltrating tumor cells (Fig. 6, panel C"). These data reveal a transitional shift in galectin-3 expression and distribution with breast cancer progression and suggest acquisition and/or assumption of novel galectin-3 mediated functional interactions with the stromal microenvironment that are probably necessary for progression.

Discussion

Galectin-3, a member of the β-galactoside-binding lectin family, has been implicated in several biological events and plays a role in tumor progression and metastasis by regulating homotypic and heterotypic cell-cell interactions and cell-matrix interactions. Galectin-3 has been positively and negatively correlated with tumor progression. For instance, expression of galectin-3 has been directly correlated with neoplastic potential and metastatic capacity in fibrosarcoma and melanoma cells [26], whereas galectin-3 levels were found to be downregulated in advanced prostate [13] and colon carcinomas [12,27] when compared with their respective normal counterparts. Similarly, reduced expression of galectin-3 has been reported in advanced histological grades of breast cancer [11,15]. These findings conflict with *in vitro* studies utilizing MDA-MB-435 and BT-549 breast cancer cell lines where a direct correlation between galectin-3 expression and metastatic and invasive potential was observed [28,29].

Until now majority of the studies have reported quantitative differences in galectin-3 protein expression between normal and cancerous tissues without ascribing alterations in expression to specific morphologic subtypes that are precursors for breast cancer progression. To clarify the function of galectin-3 in breast cancer progression, we have a) characterized the role of galectin-3 in establishment and maintenance of stable functional heterotypic interactions between epithelial and endothelial cells using a three-dimensional coculture system on reconstituted basement membrane [17] and b) performed detailed analysis of expression and distribution of galectin-3 mRNA and protein in human breast tissues, EIII8-HUVEC three-dimensional cocultures, and xenografts that recapitulate morphologic subtypes that are characteristic of human breast cancer evolution. Consistent with previous reports, our results show that normal and hyperplastic

ducts express elevated levels of galectin-3 mRNA and protein in the luminal epithelial cells [11], and is downregulated in early grades of DCIS. Interestingly, galectin-3 is reexpressed in the peripheral tumor cells as DCIS lesions evolve and/or progress to comedo-DCIS, the latter a precursor subtype conferring high risk for progression [30-32]. This pattern of galectin-3 mRNA and protein expression in the peripheral tumor cells is maintained in invasive breast carcinomas. These data suggest that galectin-3 expression is not only associated with specific morphologic precursor-subtypes of breast cancer but is also accompanied by a transitional switch in expression from luminal to basal epithelial cells that is coincident with acquisition of invasive potential. Such localized expression of galectin-3 in cancer cells proximal to the stroma could lead to increase in invasive potential by inducing better interactions with the stromal counterparts. Our findings are in agreement with those of [33] who have indicated that localized increase in threshold concentrations of galectin at the invasive sites is directly correlated with invasive capacity of a cell.

Previous studies have suggested that expression of galectin-3 is found in the cytosol and occasionally in the nucleus in human breast cells [11]. Expression of galectin-3 in the nucleus has been related to variations in steroid status at the time of biopsy, as estradiol and progestins have been suggested to modulate galectin-3 expression in estrogen-receptor (ER)-positive human breast cancer cells [34]. Results from our study also show occasional expression of galectin-3 in the nuclei of ER-positive estrogen responsive EIII8 cells [17,22], thus suggesting that variations in cytoplasmic versus nuclear localization of galectin-3 are probably a result of changes in hormonal status.

Upregulation in galectin-3 could allow enhanced interactions with stromal cells and hence greater adhesion to target organ endothelial cells. To understand the role of galectin-3 in mediation of functional heterotypic interactions between breast epithelial and endothelial cells, we have utilized a three-dimensional coculture system that allows establishment of reciprocal and productive interactions between EIII8 and HUVEC cells [Shekhar, 2000 #176;]. Using this system, our data from galectin-3 immunoneutralization experiments have showed that galectin-3 is necessary for maintenance and stability of heterotypic interaction networks between EIII8 and HUVEC cells. Although galectin-3 is derived mainly from the epithelial compartment, it is interesting to note that coculture of EIII8 cells with endothelial cells results in significant induction of secreted galectin-3 levels. Inclusion of modified citrus pectin caused a significant decrease in the levels of secreted galectin-3. These results are in agreement with our previous studies that demonstrated the ability of modified citrus pectin to effectively sequester galectin-3 and inhibit galectin-3 mediated functions such as homotypic tumor cell aggregation, binding of tumor endothelial cells, *in vitro* and *in vivo* tumor growth and metastasis [23].

Galectin-3 is protected from exogenous collagenases in the cells as majority of galectin-3 is found in its native 31 kDa form in EIII8-HUVEC cocultures. Galectin-3 secretion is probably slow in EIII8 cells and may account for the lower levels of secreted galectin-3 in EIII8 homotypic cultures. Interestingly, coculture with endothelial cells results in significant increase in levels of secreted 31 kDa galectin-3, which is also reflected by the presence of detectable levels of a 62 kDa galectin-3-immunoreactive band. Galectin-3 exists as a monomeric protein at sub-micromolar concentrations and forms homodimers at high concentrations [35-38], thus suggesting that the 62 kDa band may represent galectin-3 homodimers. Following release, the secreted or soluble galectin-3

exists as 31 and 27kDa forms, the latter a result of proteolytic processing at collagenase-sensitive cleavage site at amino acid residues Gly32-Ala33 (unpublished). Our previous studies have shown that a major breakdown product of galectin-3 cleavage *in vitro* by purified activated MMP-2 and MMP-9 is 22 kDa protein that ensues as a result of cleavage at Ala62-Tyr63 [39]. However, our present data indicate that secreted endogenous galectin-3 is insensitive to cleavage at Ala62-Tyr63, and that a site 5' of this previously demonstrated MMP-2/-9 sensitive site is utilized. This difference in collagenase-sensitivity observed *in vitro* with recombinant galectin-3 versus *in vivo* with endogenous galectin-3 probably arises from variations in accessibility of cleavage sites *in vivo*. Interestingly, cleavage at Ala62-Tyr63 occurs, albeit to a minor extent, inside the cells as only the 22 kDa galectin-3 immunoreactive band is detected in the cell lysates. These data suggest that differences in proteolytic processing of secreted versus intracellular galectin-3 molecules probably arise from differences in accessibility of sensitive sites, levels and/or type of activated protease(s). It is conceivable that the differentially processed galectin-3 molecules inside and outside the cells serve different functional roles since following initial cleavage, the newly generated amino terminus is not further processed. It is intriguing to speculate that the 27 kDa cleaved product is a result of proteolytic clipping by MMP-2 as we have previously demonstrated the characteristic presence of active MMP-2 in the conditioned media of only EIII8-HUVEC cocultures and not in corresponding EIII8 or HUVEC homotypic cultures [17]. Another interesting feature observed is that the ratios of secreted native 31 to cleaved 27 kDa forms are maintained at constant levels, thus indicating that only a fixed proportion of secreted galectin-3 is accessible for proteolytic processing. It is not clear at present how galectin-3 lacking the collagen-like amino terminal domain influences galectin-3 interaction as the amino terminal end has been proposed to mediate galectin-3 dimerization [36,40].

In summary, our findings indicate that galectin-3 mRNA and protein expression is not only associated with specific morphologic precursor-subtypes of breast cancer but also demonstrate alterations in expression from luminal to basal epithelial cells that is coincident with acquisition of invasive potential. These data along with those obtained from breast epithelial-endothelial coculture model reinforce the importance of galectin-3 in stromal-epithelial interactions and its utility as a marker for breast cancer progression and metastasis.

Acknowledgements. This work was supported by grants DAMD 17-02-I-0618 (to MPVS) and DAMD 17-00-I-0497 (to AR) from the U.S. Army Medical Research and Materiel Command.

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Figure Legends

Fig. 1. Three-dimensional EIII8-HUVEC cocultures show up-regulated expression of secreted galectin-3 and differences in protease-sensitivity between secreted and intracellular galectin-3. Twenty or 50 μg of protein present in culture media and corresponding matrix fractions, respectively, at day 5 were analyzed by Western blotting. A, B, galectin-3 levels in conditioned media of EIII8, or EIII8-HUVEC cocultures that were untreated (control), or treated with 0.01 or 0.1% control citrus pectin (CP) or modified citrus pectin (MCP). A', B', galectin-3 detected in lysates of untreated, CP-treated and MCP-treated EIII8-HUVEC cocultures and EIII8 homotypic cultures. A, A', galectin-3 detected with polyclonal HL-31 and B, B', detection with monoclonal TIB-166 galectin-3 antibody. Arrows indicate positions of 62 (A), 27 (A) and 22 (A') kDa bands.

Fig. 2. Regulation of three-dimensional growth of EIII8-HUVEC cocultures by galectin-3. Control cultures were untreated (control) or 24 h after seeding treated with 0.5 or 1.0 μg of HL-31 or TIB166, or 1 μg of corresponding rat or rabbit nonimmune IgG. Cultures were treated with dispase to solubilize Matrigel and trypan blue excluded cells were counted by hemocytometer. Results obtained from three independent experiments performed in triplicate are expressed as mean ± SE, * ($P<0.001$), ** ($P<0.005$) amounts of antibody that inhibited cell number significantly over corresponding control.

Fig. 3. Formalin-fixed, paraffin-embedded sections of EIII8-HUVEC three-dimensional cocultures were either stained with H&E (A and B) or with antibodies to cd31 (C), factor VIII (D), cytokeratins (E), PCNA (F), or galectin-3 monoclonal TIB-166 antibody (G and H). Note the widespread immunoreactivity to cytokeratins and in the branching end buds as opposed to the localized cd31 and factor VIII8 expressing endothelial cells. Also note

the presence numerous proliferating cells in branching end buds invading into the surrounding ECM. Note that galectin-3 staining is exclusively localized in epithelial buds with higher TIB-166 reactivities in epithelial cells juxtaposed to endothelial cells (arrows). A-F, H, X10; G, X4.

Fig. 4. Galectin-3 mRNA synthesis occurs only in the epithelial compartment of EIII8-HUVEC cocultures. A and B, *in situ* hybridization analysis with antisense and sense DIG-labeled galectin-3 RNA probes, respectively. Note the presence of galectin-3 mRNA signals detected only with antisense galectin-3 probe and its exclusive presence in the epithelial compartment. Magnification, X25.

Fig. 5. Galectin-3 mRNA and protein expression in premalignant lesions produced by MCF10AT1 xenografts. A and B, *in situ* hybridization with DIG-labeled antisense and sense galectin-3 RNA probes, respectively; C, galectin-3 immunoreactivity to TIB-166 anti-galectin-3 antibody. Note the presence of strong galectin-3 protein expression in normal (thin arrow) and hyperplastic (block arrow) ducts. Also, note the presence of galectin-3 staining in the lumens of normal and hyperplastic ducts. Magnification, X4.

Fig. 6. Galectin-3 mRNA and protein expression in human breast tumors. A-C and A'-C', *in situ* hybridization with antisense and sense galectin-3 RNA probes, respectively; A"-C", galectin-3 protein staining with TIB-166 anti-galectin-3 antibody. A, A', A", normal areas of breast cancer tissue, B, B', B", papillary hyperplasias, and C, C', C", infiltrating carcinoma cells. Note the mRNA signals detected only with antisense galectin-3 probe (A-C). Also note the presence of intense galectin-3 protein immunoreactivity in the lumens of normal and hyperplastic ducts and absence of staining in the adjacent stroma (A", B"). In contrast, note the presence of very strong galectin-3 protein staining in invasive cancer cells and in fibroblasts and extracellular matrix surrounding them (C"). Magnification, A, B, A', B', A", B", X10; C, C', C", X4.

Fig. 7. Galectin-3 mRNA and protein expression exhibit alterations that are coincident with progression to comedo-DCIS of DCIS.com-derived xenografts. A, D, DCIS lesions harvested at 20 days; B, C, F, lesions harvested at 60 days; E, lesions harvested at 40 days. A-C, *in situ* hybridization analysis with antisense galectin-3 RNA probe; D-F, galectin-3 protein detected with TIB-166 galectin-3 antibody. Note the absence of detectable galectin-3 mRNA signals in early DCIS lesions, i.e., prior to manifestation of comedo necrotic core (A). However, upon progression to comedo-type, note the expression of galectin-3 mRNA in tumor cells away from the central necrotic core (B and C). Similarly, note that only very few tumor cells express galectin-3 protein in early DCIS lesions (D; arrow). By day 40, galectin-3 protein staining peripheral cells are prevalent (E), and upon manifestation of central comedo-necrosis, focally intense galectin-3 immunoreactive signals are observed in the tumor cells that are in close proximity to the stroma (F). A, C, D, E, X10; B, F, X4.

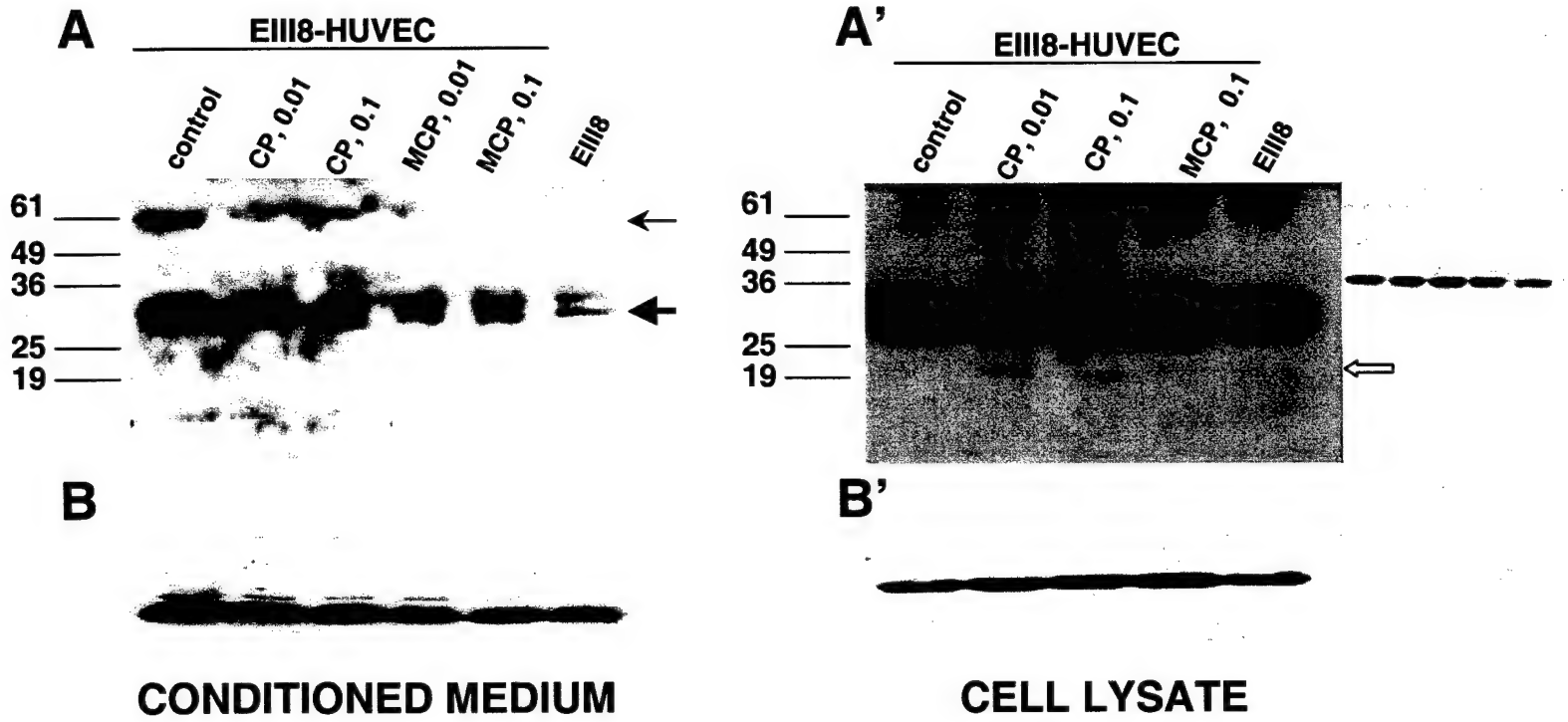
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Fig. 1

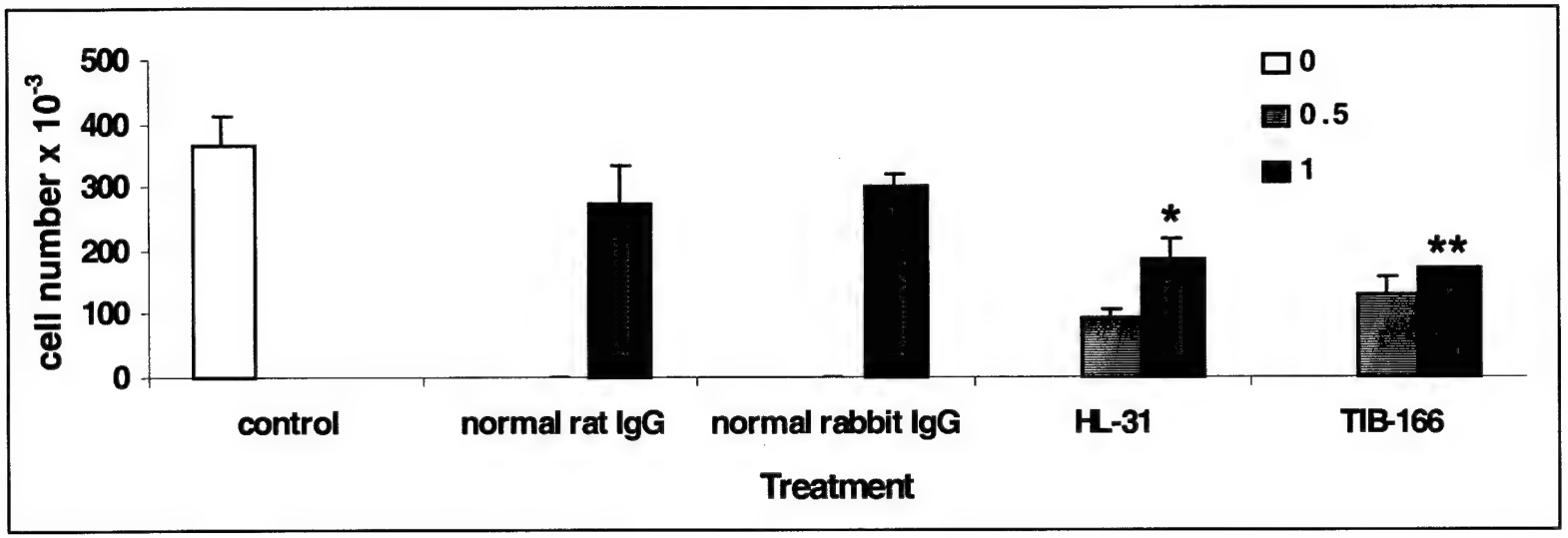
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Fig. 2

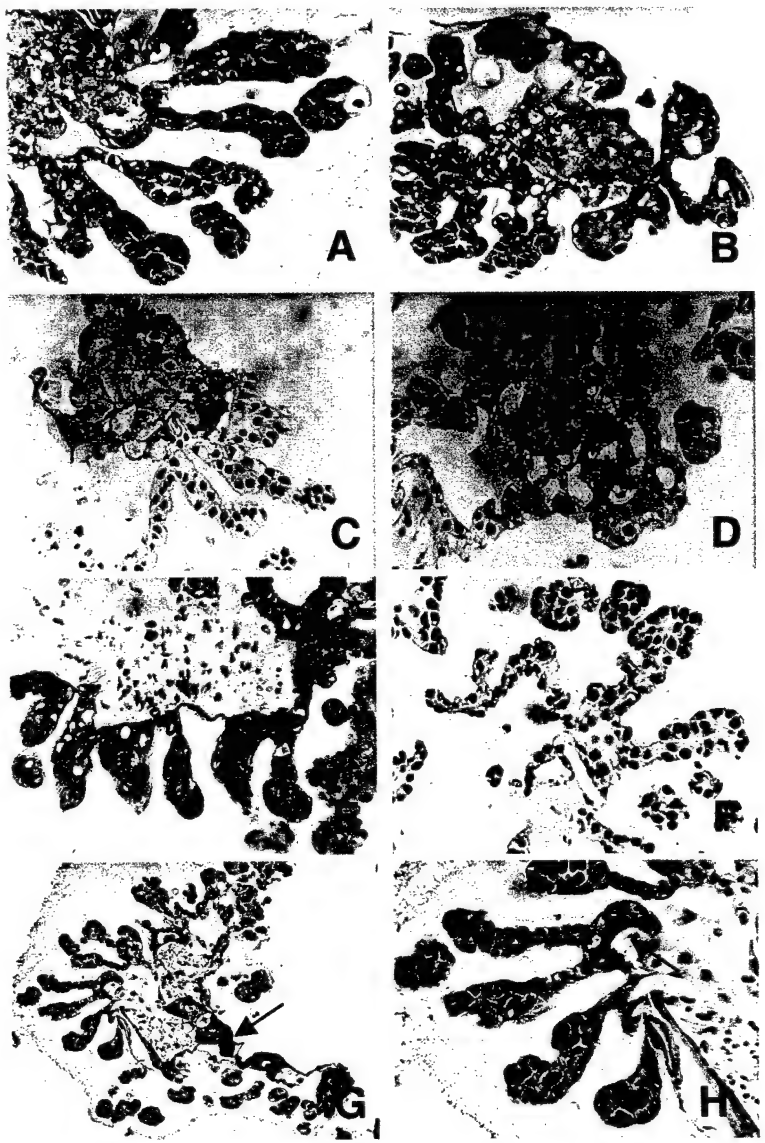

Fig. 3

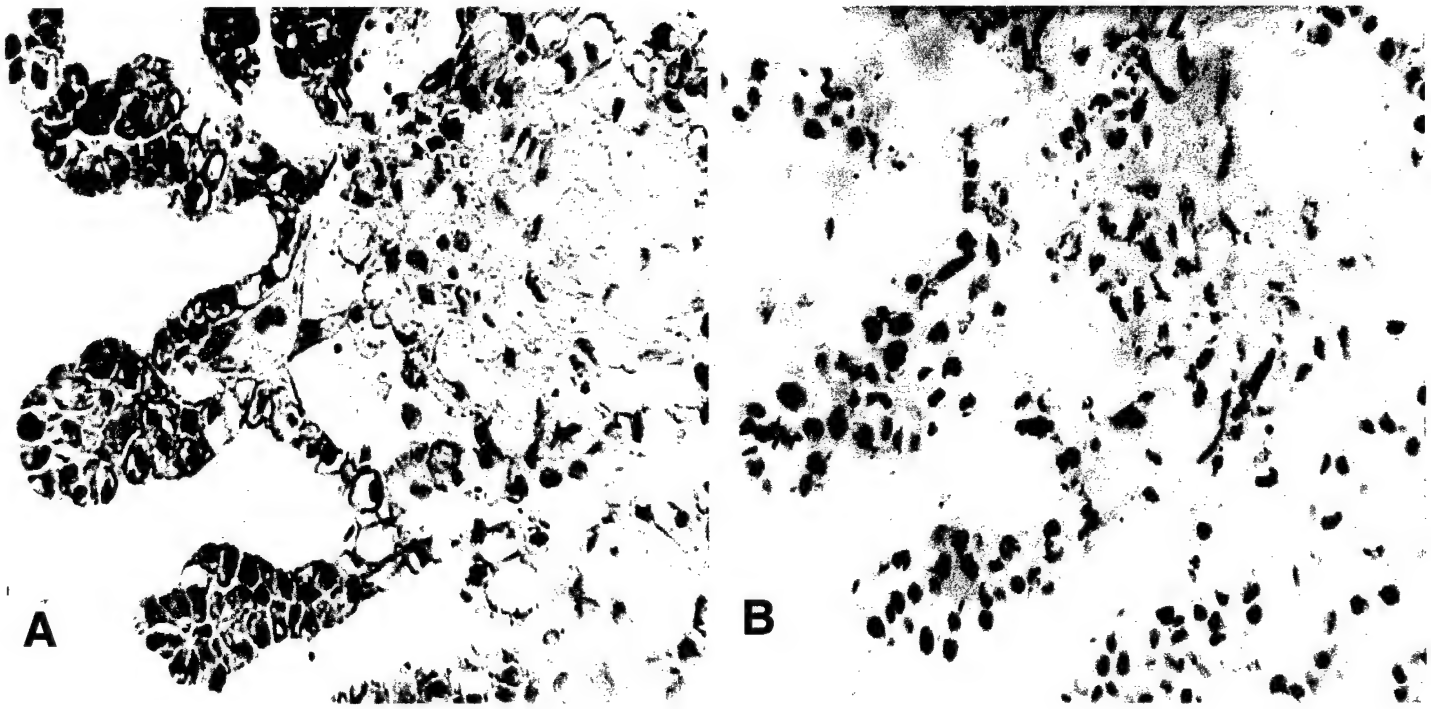

Fig. 4

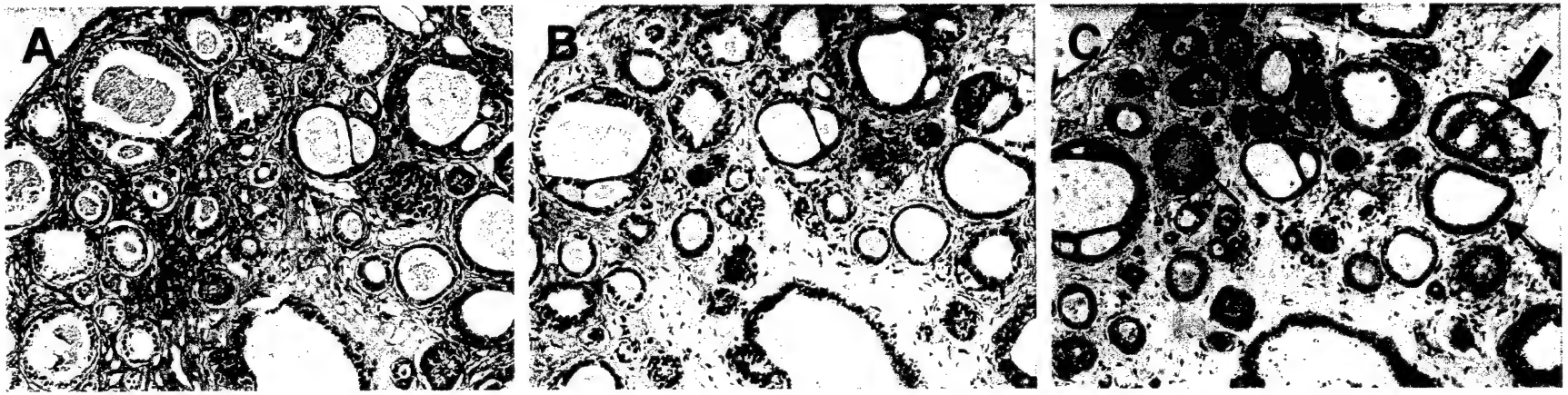

Fig. 5

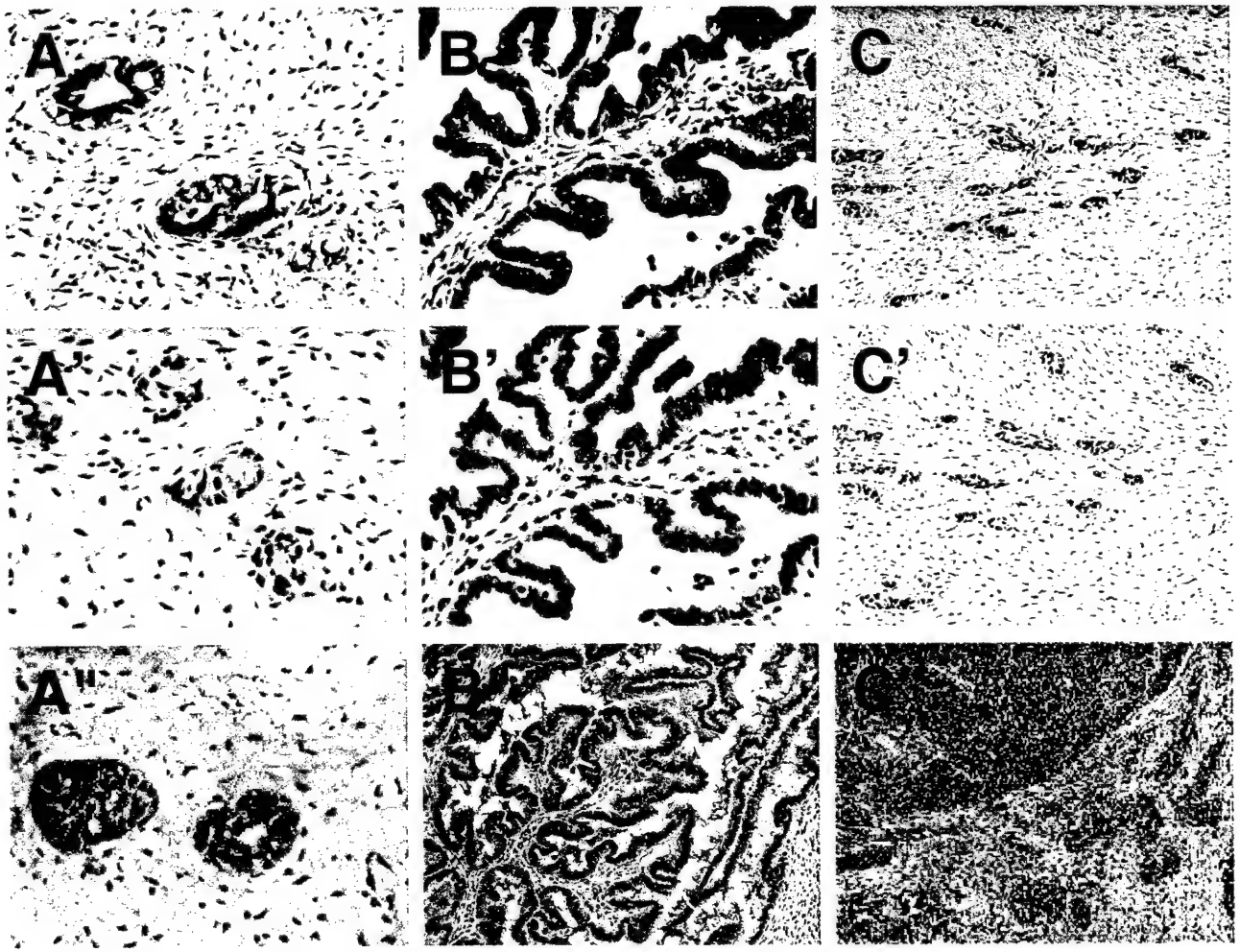

Fig. 6

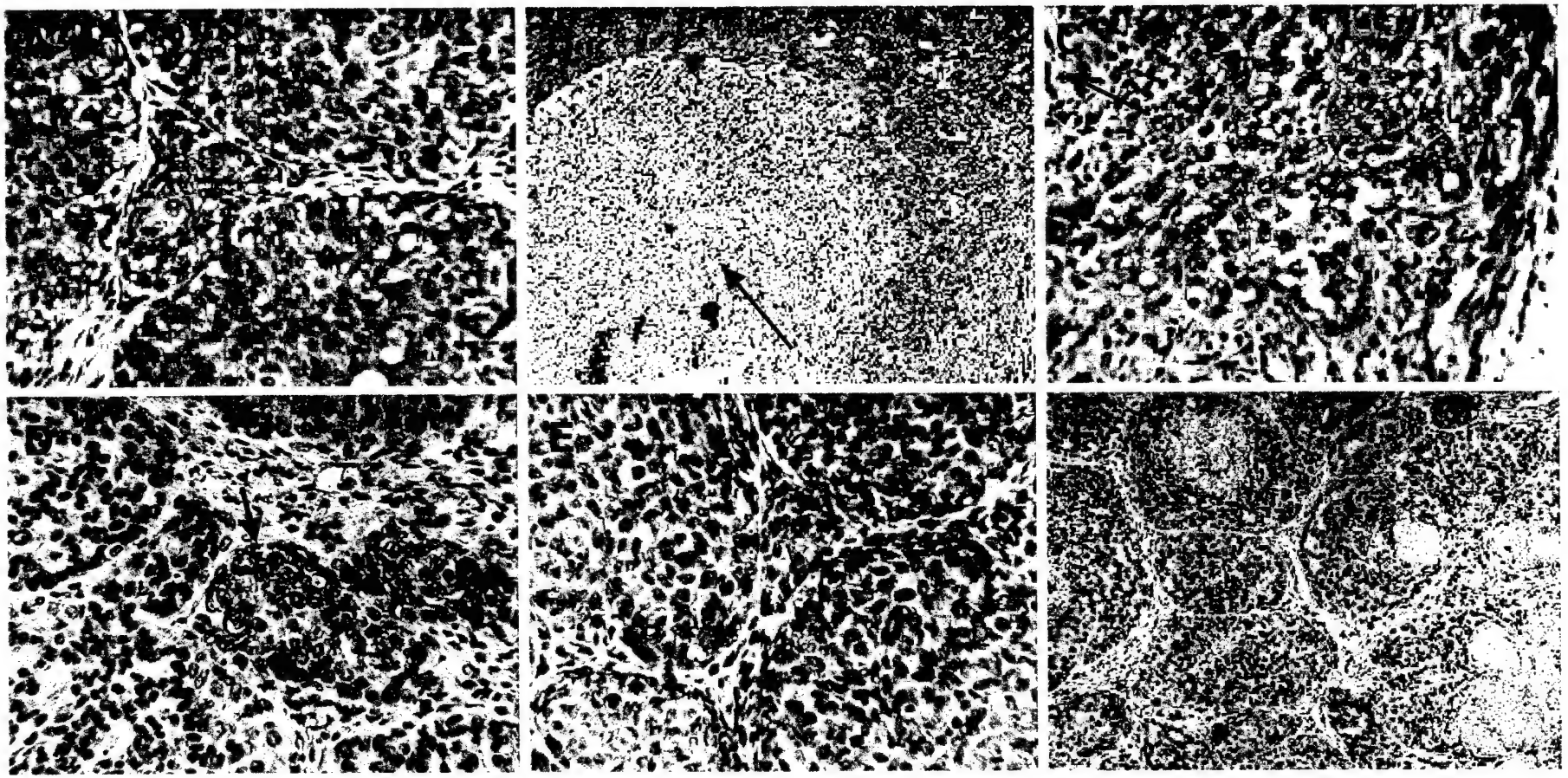

Fig. 7

Vol. 7, 661–668, March 2001

Down-Regulation of Galectin-3 Suppresses Tumorigenicity of Human Breast Carcinoma Cells[1]

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ABSTRACT

Galectin-3 is an endogenous β-galactoside-binding protein with specificity for type I and II ABH blood group epitopes and poly-*N*-acetyllactosamine glycan-containing cell surface glycoproteins and is the major nonintegrin cellular laminin-binding protein. Galectin-3 is expressed at an elevated level in a wide range of neoplasms, and expression was shown to be associated in some tumor cell systems with metastases. Here we determined the functional consequence of blocking galectin-3 expression in highly malignant human breast carcinoma MDA-MB-435 cells. Inhibition of galectin-3 expression led to reversion of the transformed phenotype as determined by altered morphology, loss of serum-independent growth, acquisition of growth inhibition properties by cell contact, and abrogation of anchorage-independent growth. The blockage of galectin-3 expression led to a significant suppression of tumor growth in nude mice. These results provide direct evidence that galectin-3 expression is necessary for the maintenance of the transformed and tumorigenic phenotype of MDA-MB-435 breast carcinoma cells.

INTRODUCTION

Galectin-3 is a member of a growing family of multifunctional galactoside-binding proteins. Members of this gene family share affinity for β-galactoside-containing glycoconjugates. Galectin-3 is a $M_r \sim 30{,}000$ protein composed of three distinct structural domains: (*a*) a short NH_2 terminus of 12 amino acids that controls its cellular targeting; (*b*) a repetitive collagen-like sequence rich in glycine, tyrosine, and proline, which serves as a substrate for matrix metalloproteinases; and (*c*) the COOH-terminal domain, a globular structure encompassing the carbohydrate-binding site (1–4). In some human tumors, a direct relationship was shown between galectin-3 levels and the stage of tumor progression (5–11). We have recently reported (9) that the expression of galectin-3 is related to neoplastic transformation and progression toward metastasis in colon carcinoma. In gastric carcinoma, it was found that tissue levels of galectin-3 were higher in certain primary tumors and their metastases than in the adjacent normal mucosa (7). In ovarian carcinoma, however, no correlation was observed between galectin-3 expression and clinicopathological features (12). In steroid-sensitive breast carcinoma cells, it was suggested that estradiol and progestin might act as coordinates regulating specific genes, including up-regulation of galectin-3 expression, leading to the acquisition of metastatic phenotype (13). Previously, we studied galectin-3 expression in cultured human breast carcinoma cell lines characterized as nontumorigenic, poorly metastatic, or metastatic in nude mice, and the expression of galectin-3 correlated with the reported tumorigenicity of the cells (14). The introduction of rgalectin-3[4] into null-expressing nontumorigenic BT-549 cells resulted in the acquisition of anchorage-independent growth properties and tumorigenicity, suggesting a relationship between galectin-3 expression and malignancy of human breast carcinoma cell lines (14). Down-regulation of galectin-3 expression by colon carcinoma cells resulted in a significant decease in liver colonization ability, whereas up-regulation of galectin-3 increased metastatic potential (15). These findings imply an involvement of galectin-3 in malignant progression of carcinomas and suggest a possibility that galectin-3 may serve as a potential molecular target for therapy of carcinomas harboring overexpressed galectin-3. Thus, the present study was designed to directly examine and establish the role of galectin-3 in breast cancer. To this end, we blocked galectin-3 expression in the highly tumorigenic MDA-MB-435 human breast carcinoma cell line by antisense transfection and analyzed the effect of down-regulation of galectin-3 expression on cellular phenotypes associated with transformation *in vitro* and *in vivo*.

MATERIALS AND METHODS

Cell Culture. The human breast cancer cell line MDA-MB-435 was grown as described previously (14) in monolayer culture in DMEM (Life Technologies, Inc., Grand Island, NY) supplemented with 10% heat-inactivated FBS, essential and nonessential amino acids, vitamins, penicillin, and streptomycin (Life Technologies, Inc.). The culture was maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Derivative

Received 10/26/00; revised 12/11/00; accepted 12/13/00.
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[1] Supported in part by NIH Grant CA46120 from the National Cancer Institute and by Department of Defense Grant BC991100 from the U.S. Army Medical Research and Materiel Command.
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[4] The abbreviation used is: rgalectin-3, recombinant galectin-3; FBS, fetal bovine serum; MFP, mammary fat pad.

cell lines were also grown under these conditions; however, the medium was supplemented with 1.2 μg/ml Geneticin (G418).

Plasmid Constructs. The second intron of the galectin-3 gene containing 649 bp flanked by 28 bp of the second exon on the 5′ end and 30 bp of the third exon on the 3′ end was amplified by PCR from genomic DNA of MDA-MB-435. The sense primer used was 5′-CGAGCGGAAAATGGCAGACAA-3′, and the antisense primer used was 5′-GTTTCCAGACCCA-GATAACG-3′. Appropriately sized (0.7 kb) PCR product was ligated into pGEM-T vector (Promega, Madison, WI), which was then transformed into JM109 competent cells (Promega). An *Eco*RI-*Eco*RI fragment was subcloned into eukaryotic expression vector pCNC10 (a generous gift from Dr. F. G. Kern; Lombardi Cancer Research Center, Washington, D.C.) in the antisense orientation downstream of the cytomegalovirus promoter. A clone was selected for orientation by restriction digestion analysis and direct DNA sequencing and was designated pCNC10-G-AS.

Transfection. MDA-MB-435 human breast carcinoma cells were transfected with either the control (pCNC10) or antisense (pCNC10-G-AS) plasmid DNA by LipofectAMINE reagent (Life Technologies, Inc.) according to the supplier's protocol. Briefly, 2 μg of plasmid DNA were incubated with 20 μl of LipofectAMINE reagent and 400 μl of serum-free DMEM at room temperature for 45 min. The mixture was added to cells grown to 70% confluence in a 35-mm dish in 2 ml of serum-free DMEM and incubated at 37°C for 5 h, following which DMEM containing 20% serum was added to the cells. After incubation for 48 h, G418 was added to the medium to a concentration of 800 μg/ml, and the cells were maintained for 2 weeks. The G418-resistant clones were subcloned by cloning cylinders, and two clones, MDA-MB-435-AS1 and MDA-MB-435-AS2, were selected as antisense transfectants for subsequent experiments. MDA-MB-435-CONT, a control transfectant, was also obtained. Stable integration of the plasmid DNA into the transfectants was verified by Southern blot analysis as described below.

Southern Blot Analysis. Genomic DNA (15 μg) was digested with *Eco*RI (Promega) for 2 h at 37°C. The resulting fragments were electrophoresed in 0.7% agarose gel, transferred to Magna Charge nylon membrane (Micron Separation, Inc., Westborough, MA), and UV cross-linked. The membrane was prehybridized at 42°C for 4 h with a solution containing 50% formamide, 5% dextran sulfate, 5× Denhardt's solution, 50 mM sodium phosphate, 5× SSC, and 300 μg/ml denatured salmon sperm DNA. Hybridizations were preformed with a randomly primed ^{32}P-labeled 707-bp DNA insert from the original plasmid clone as a probe (Ready-To-Go DNA labeling beads; Pharmacia). The membranes were washed at 55°C with 2× SSC and 0.1% SDS for 30 min, followed by a wash with 0.2× SSC and 0.1% SDS for 30 min. The extent of hybridization was visualized by exposure to X-ray film (Eastman Kodak, Rochester, NY).

Northern Blot Analysis. Total cellular RNA was isolated from cultured cell lines as described previously (14). Twenty μg of total RNA were separated on 1% denaturing formaldehyde-agarose gels, transferred to Magna Charge nylon membrane, and UV cross-linked. Prehybridization was performed in the same way as described for the Southern blot. Hybridizations were performed with a randomly primed ^{32}P-labeled probe (Ready-To-Go DNA labeling beads) representing the entire open reading frame of the galectin-3 cDNA. The membrane was washed twice, and the extent of hybridization was visualized as described above. Human β-actin was also used as a probe for normalization.

Western Analysis. Cells were grown to 80% confluence, and whole-cell lysates were prepared in lysis buffer [20 mM Tris-HCl (pH 7.4), 0.1% SDS (Fisher Scientific, Pittsburgh, PA), 1.0% Triton X-100, 1.0% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, and 1 μg/ml aprotinin (Sigma, St. Louis, MO)]. Equal volumes of each lysate were resolved by 12.5% SDS-PAGE and electroblotted onto polyvinylidene difluoride plus membrane (Micron Separation, Inc.). Membranes were quenched in a solution of PBS containing 5% nonfat dry milk and 0.1% Tween 20 for 30 min on a rotary shaker. Blots were incubated with a 1:500 dilution of rat anti-galectin monoclonal solution for 1 h. After washing three times (for 10 min each) in quench solution, membranes were incubated with a 1:5000 dilution of anti-rat horseradish peroxidase-conjugated secondary antibody (Zymed, South San Francisco, CA) in quench solution for 1 h, washed three times (for 10 min each) in quench solution, and then visualized by exposure for 1 min to a freshly prepared chemiluminescent substrate containing 0.0624 mM luminol (3-aminoptalhydrazide; Sigma), 100 mM *p*- coumaric acid (Sigma), and 1.4 mM H_2O_2 (Fisher Chemical, Fair Lawn, NJ) in 5 mM Tris buffer (Sigma; pH 8.5). Excess liquid was removed by touching the corner of the membrane to Whatman 3 MM Chr paper, (Whatman International Ltd., Maidstone, United Kingdom). The membrane was then wrapped in Saran Wrap and exposed to Kodak Biomax-MJR film (Eastman Kodak) for 1 min. The bands were quantified on Kodak Image Station 440CF using ID Image Software (Eastman Kodak).

rGalectin-3. Human rgalectin-3 was isolated from bacterial cells by affinity chromatography on asialofetuin-Affi-Gel15 (Bio-Rad, Hercules, CA) as described previously (16), followed by extensive dialysis against PBS (pH 7.4). Subsequently, PBS was replaced with DMEM using Ultrafree-MC filters (Millipore, Bedford, MA).

Assessment of Saturation Density and Cell Morphology. Logarithmically growing cells were plated at a density of 5×10^3 cells/cm^2 onto 60-mm culture dishes. The tissue culture medium was replaced daily to ensure that growth arrest would be a function of cell density rather than medium depletion. Cells were observed morphologically before trypsinization. Phase-contrast microscopy was used to obtain photographs. Viable cell counts were determined daily by trypan blue dye exclusion.

Growth in Monolayer Culture. Cells (1×10^5) were seeded onto 60-mm culture dishes in the normal medium with four different serum concentrations (10%, 5%, 2.5%, and 1.25%). The tissue culture medium was changed every day to avoid medium depletion. Viable cell counts were determined daily using a hemocytometer and trypan blue dye exclusion. In another set of experiments, rgalectin-3 was added to the medium at a concentration of 10 μg/ml. Each experiment was performed in triplicate.

Immunofluorescence Labeling. Cells were detached from 100-mm plates with 0.25% trypsin-EDTA (Life Technologies, Inc.) and washed twice with PBS containing Ca^{2+} and

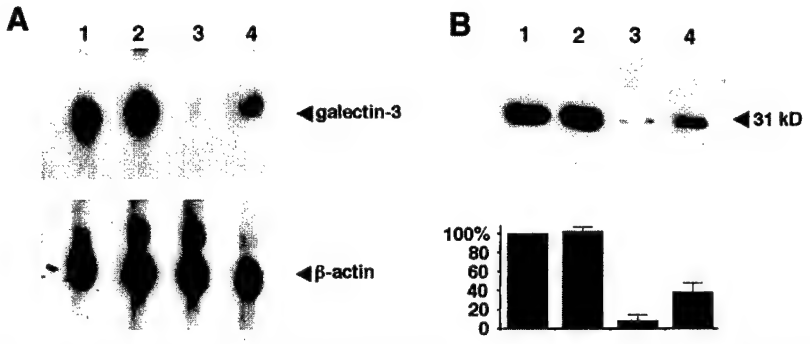

Fig. 1 Expression of galectin-3 in transfected clones. *A*, Northern blot analysis. Twenty μg of RNA were electrophoresed through a 1% denaturating formaldehyde-agarose gel, transferred to nylon membrane, and hybridized to the ^{32}P-labeled galectin-3 cDNA (*top panel*). Rehybridization of same membrane was performed with ^{32}P-labeled β-actin to confirm that each lane contained the same amount of RNA (*bottom panel*). *B*, Western blot analysis. Fifty-μg protein samples from cell lysate were subjected to reducing 12.5% SDS-PAGE, transferred to polyvinylidene difluoride membrane, and probed with rat anti-galectin-3 antibody (*top panel*). The signals were scanned and calculated using ID Image Analysis Software (*bottom panel*). The experiment was performed in triplicate using different protein preparations. Each histogram represents the mean of densitometry integration as a percentage of parental cell intensity. *Lane 1*, parental MDA-MB-435; *Lane 2*, vector only-transfected MDA-MB-435-CONT; *Lanes 3* and *4*, galectin-3 antisense-transfected MDA-MB-435-AS1 and MDA-MB-435-AS2, respectively.

Mg^{2+}. Coverslips sterilized with methanol were placed into 6-well plates; 1×10^5 cells were seeded into each well, and the cells were grown overnight in 10% DMEM. The cells were rinsed twice with PBS; for cell surface staining, they were fixed with 3.5% paraformaldehyde for 5 min on ice and 5 min at room temperature; for intracellular staining, cells were fixed and permeablized with 100% methanol for 15 min at room temperature. Cells were then washed twice with PBS and blocked with 1% BSA in PBS. After a 30-min incubation on ice, rabbit galectin-3 antiserum was added at a 1:50 dilution in 1% BSA and incubated for 1 h at room temperature, followed by three washes with PBS. The following steps were performed in the dark. Secondary antibody (FITC goat antirabbit IgG; Zymed; Ref. 14) was added at a 1:200 dilution in 1% BSA, incubated for 1 h, and then washed three times with PBS. The coverslips were transferred upside down onto glass slides with one drop of polyvinyl alcohol in PBS. Slides were then wrapped with aluminum foil and stored at 4°C until visualization.

Anchorage-independent Growth and Tumorigenicity. Assays were performed in 6-well dishes coated with 1% agarose dissolved in DMEM supplemented with 10% FBS. One thousand cells uniformly suspended in 0.5% agarose/DMEM were overlaid on the bottom layer. The dishes were kept at 4°C for 2 h to solidify the agarose and incubated at 37°C. After 24 h, 2 ml of fresh medium were placed on agar. The medium was replaced every 2 days. After 14 days, the number of colonies was counted and photographed using phase-contrast photomicrography. Colonies measuring 0.1 mm in diameter or greater were scored. Results were expressed as the percentage of colonies formed per total number of seeded cells. In another set of experiments, rgalectin-3 (10 μg/ml) was added to the medium. Each experiment was performed in triplicate.

To determine *in vivo* tumorigenicity, 5×10^5 or 1×10^5 cells in a total volume of 0.1 ml of PBS were injected into the MFP region of 6-week-old athymic female nude mice. The growth of MFP tumors was monitored by weekly examination, and growth rates were determined by caliper measurements of width and length.

Statistics. All data points represent the mean ± SE. Statistical analysis was performed by using the Mann-Whitney test.

RESULTS

Inhibition of Galectin-3 Expression in MDA-MB-435 Antisense Transfectants. To better understand whether galectin-3 plays a functional role in breast cancer, human breast carcinoma MDA-MB-435 cells were transfected with antisense galectin-3 and control vectors, and the biological consequence of down-regulation of galectin-3 was evaluated. Individual G418-resistant cell clones were isolated, and stable integration of expression constructs was verified by Southern blot analysis. The expression levels of galectin-3 mRNA were determined by Northern blot analysis using full-length galectin-3 cDNA as a probe, and because the antisense galectin-3 DNA construct was transcribed only from the second intron together with the flanking regions, this analysis would detect only endogenous galectin-3 mRNA, and not antisense RNA. Fig. 1*A* reveals that the control transfection did not alter galectin-3 expression, whereas introduction of galectin-3 antisense into MDA-MB-435 resulted in a marked reduction in the expression of galectin-3-specific mRNA, ranging from >90 to ~50% inhibition. Two representatives clones are depicted in Fig. 1*A*, *Lanes 3* and *4*, respectively. These changes in mRNA levels were accompanied by corresponding decreases in galectin-3 protein levels (Fig. 1*B*). Cells of representative clones MDA-MB-435-AS1 and MDA-MB-435-AS2 showed a reduction of galectin-3 expression by 91.9% and 61.8%, respectively, as compared with parental MDA-MB-435 cells (Fig. 1*B*, *bottom panel*).

***In Vitro* Growth Properties and Tumorigenicity Associated with Galectin-3 Down-Regulation.** Inhibition of galectin-3 expression led to a marked alteration in cell morphology. Fig. 2 depicts the morphology of the cells after 2 (Fig. 2, *left panels*) and 7 days (Fig. 2, *right panels*) days in culture. At low cell density, parental MDA-MB-435 (Fig. 2*A*) and MDA-MB-435-CONT cells (Fig. 2*C*) showed a similar retractile,

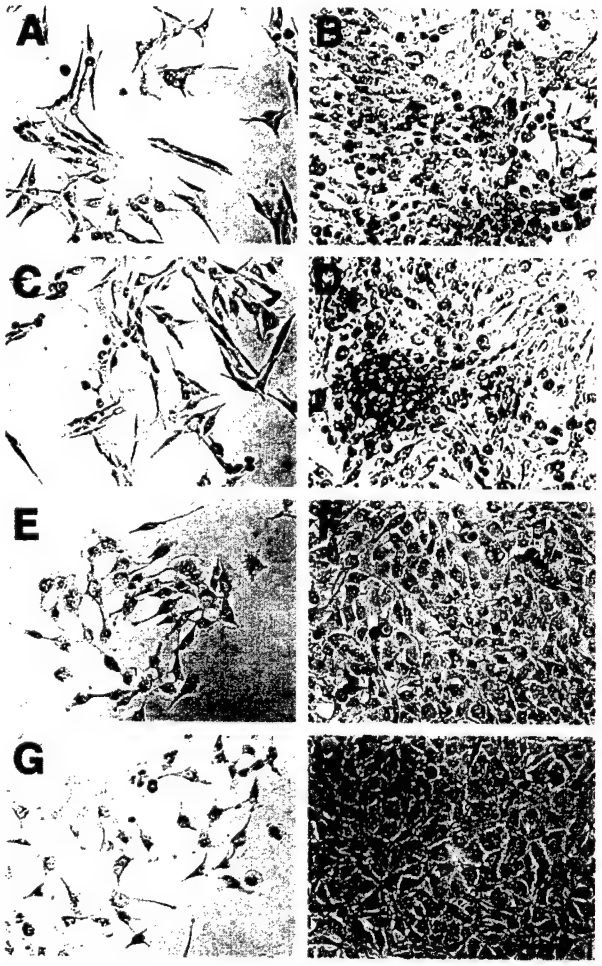

Fig. 2 Morphology of MDA-MB-435 cells and transfectants. MDA-MB-435, *A* and *B*; MDA-MB-435-CONT, *C* and *D*; MDA-MB-435-AS1, *E* and *F*; MDA-MB-435-AS2, *G* and *H*. Cells (1×10^5) were plated onto 60-mm tissue culture dishes in media supplemented with 10% FBS and incubated in the CO_2 incubator under the conditions described in "Materials and Methods." Cells were photographed by phase-contrast microscopy after 2 (*A*, *C*, *E*, and *G*) or 7 days (*B*, *D*, *F*, and *H*) of growth. All fields were examined at the same magnification. *Bar*, 50 μm.

spindle-shaped morphology, whereas MDA-MB-435-AS1 (Fig. 2*E*) and MDA-MB-435-AS2 (Fig. 2*G*) cells exhibited a highly spread flat morphology with an increased cytoplasm:nuclear volume ratio. At high density, MDA-MB-435 (Fig. 2B) and MDA-MB-435-CONT cells (Fig. 2D) grew as multilayered sheets, forming a three-dimensional foci. In contrast, MDA-MB-435-AS1 (Fig. 2*F*) and MDA-MB-435-AS2 cells (Fig. 2H) remained as a uniform monolayer with a cobblestone-like pattern. Furthermore, the highest cell densities reached by MDA-MB-435 parental and MDA-MB-435-CONT cells were 4.2×10^2 and 4.1×10^2 cells/cm^2, respectively. MDA-MB-435-AS1 and MDA-MB-435-AS2 cells reached saturation at a density as low as 3.0×10^2 and 3.3×10^2 cells/cm^2, respectively. Next we tested the response of the cells to a reduced serum concentration in the growing medium (Fig. 3). At relatively high serum concentrations of 10% and 5%, the proliferation rate of all four cell lines tested was similar (Fig. 3, *A* and *B*). However, when the serum concentration was reduced to 2.5% or 1.25% (Fig. 3, *C* and *D*), MDA-MB-435 and MDA-MB-435-CONT cells maintained their normal growth rate, whereas the reduced level of galectin-3 expression was associated with a significant reduction in the growth rate of the cells in antisense clones (Fig. 3, *C* and *D*). The addition of exogenous rgalectin-3 (10 μg/ml) to the culture medium did not affect either the cell morphology or growth rate of any of the cell lines (data not shown), suggesting that the observed effects of blockage of galectin-3 are intracellular.

Next we analyzed the clonogenic and growth properties in semisolid medium. From the data summarized in Fig. 4, it is apparent that colony-forming efficiency was markedly reduced by antisense inhibition by galectin-3 expression. The number of colonies developed by MDA-MB-435-AS1 or MDA-MB-435-AS2 cells was much lower than that of MDA-MB-435 or MDA-MB-435-CONT cells (Fig. 4, compare *C* and *D* to *A* and *B*). The reduction in colony formation was not restricted to cell number but also applied to size. As shown in Fig. 4, MDA-MB-435 and MDA-MB-435-CONT cells grew to form similarly large-size colonies (Fig. 4, *A* and *B*), whereas the colony size of MDA-MB-435-AS1 was markedly smaller (Fig. 4, *C* and *D*) and that of MDA-MB-435-AS2 seemed intermediate (Fig. 4*D*). Anchorage-independent growth *in vitro* serves as a hallmark for cell transformation and may be correlated with tumorigenicity *in vivo* (17, 18). Thus, we questioned whether the reduction in galectin-3 expression might also be reflected in altered tumorigenicity in nude mice. To address this, we injected equal amounts of each cell line into the MFP of female nude mice, the preferred site of growth for MDA-MB-435 cells (19). Injection of 1×10^5 MDA-MB-435 and MDA-MB-435-CONT cells led to the formation of visible tumors within 3–4 weeks in all mice, whereas no tumor developed for up to 10 weeks in mice that received injection of an equal number of MDA-MB-435-AS1 or MDA-MB-435-AS2 cells (Table 1; Fig. 5*A*). When the inoculum was increased to 5×10^5 cells, again all of the mice injected with MDA-MB-435 or MDA-MB-435-CONT cells rapidly developed tumors. In contrast, only 3 of 10 mice injected with MDA-MB-435-AS1 or MDA-MB-435-AS2 cells developed slow-growing tumors (Fig. 5*B*). The rest of the mice remained tumor free for the 10-week duration of the experiments (Table 1; Fig. 5*B*). At the termination of experiments, the tumors developed by each cell line were removed and dissociated, and cells were regrown in culture to determine whether any change in galectin-3 expression might occur during tumor development in mice, and whether the cells have their original phenotype (data not shown).

Cellular Localization of Galectin-3. The spatial cellular localization of galectin-3 was established by immunostaining of both controls and antisense-transfected cells using anti-galectin-3 antibodies (Fig. 6). Cytoplasmic and nuclear localization of galectin-3 was readily visualized in the parental (Fig. 6*A*) and control-transfected cells (Fig. 6*B*). The galectin-3 staining was greatly diminished in MDA-435-AS1 and MDA-435-AS2 cells, and no nuclear deposition could be detected in cells containing the galectin-3 antisense sequence (Fig. 6, *C* and *D*). Control labeling of the cells with only the secondary antibody was negative (data not shown).

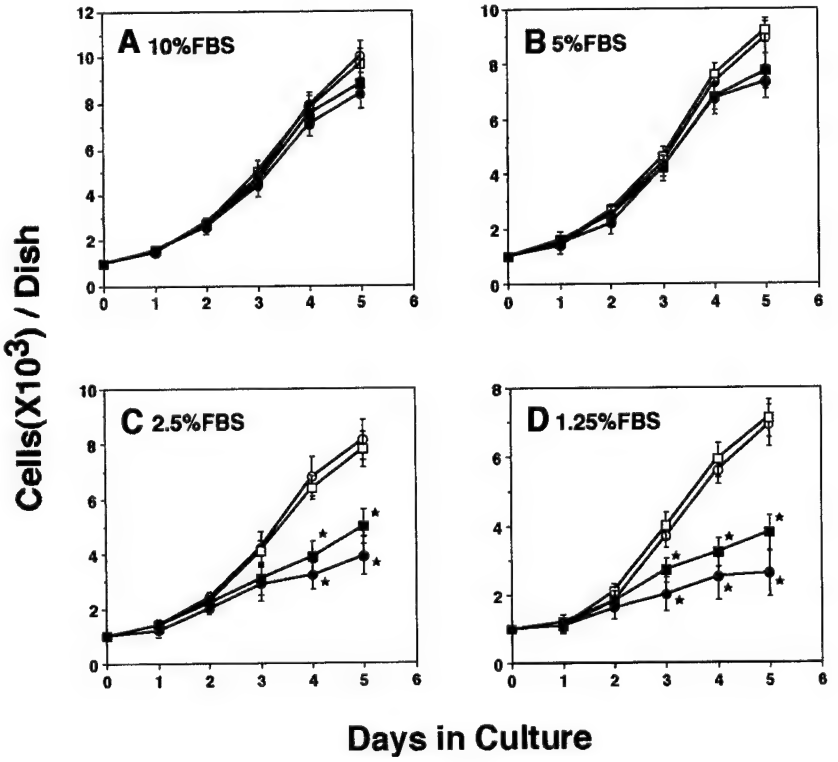

Fig. 3 Monolayer growth rate of MDA-MB-435 (○), MDA-MB-435-CONT (□), MDA-MB-435-AS1(●), and MDA-MB-435-AS2 (■). Cells (1×10^5) were plated onto 60-mm tissue culture dishes in DMEM supplemented with four different serum concentrations [10% FBS (*A*), 5% FBS (*B*), 2.5% FBS (*C*), and 1.25% FBS (*D*)]. Cells were harvested and counted in a hemocytometer at 24-h intervals. The results represent the average ± SD of three experiments, each performed in triplicate. *, $P < 0.05$ as compared with parental MDA-MB-435 cells by Mann-Whitney test.

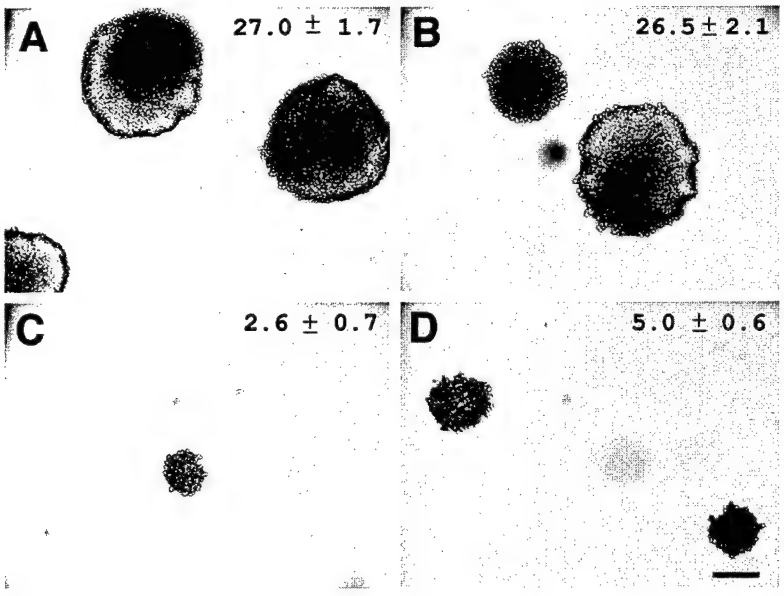

Fig. 4 Cell growth in soft agar. Cells (1×10^3) were seeded in 0.5% agarose in media supplemented with 10% FBS on top of a 1% agarose/media basal layer in 6-well dishes in quadruplicates and incubated in the CO_2 incubator under the conditions described in "Materials and Methods." The plates were examined by phase-contrast microscopy and photographed after 14 days of growth. *A*, MDA-MB-435; *B*, MDA-MB-435-CONT; *C*, MDA-MB-435-AS1; *D*, MDA-MB-435-AS2. *Bar*, 10 μm. *Inset*, numbers represent plating efficiency (percentage) after 2 weeks in culture.

DISCUSSION

The data presented herein suggest that blockage of galectin-3 expression in MDA-MB-435 breast carcinoma cells leads to partial reversion of the transformed phenotype *in vitro* and to a significant reduction in tumorigenicity *in vivo*.

Galectin-3 is a member of the galectin gene family that is expressed at elevated levels in a variety of neoplastic cell types (4–11), and it has been associated with alterations in cell growth, transformation, and metastasis (16, 20–22). Although the precise biological function of galectin-3 is unknown, its expression has been associated with events that may promote tumor progression and metastasis, such as carbohydrate-mediated homotypic aggregation, angiogenesis, and inhibition of apoptosis.

We have previously analyzed galectin-3 expression in relation to the malignant phenotypes of five established and well-

Table 1 Tumorigenicity in nude mice[a]

Cell line	Tumor formation[b]	
	Cell no.	
	1×10^5	5×10^5
MDA-MB-435	5/5	10/10
MDA-MB-435-CONT	5/5	10/10
MDA-MB-435-AS1	0/5	3/10
MDA-MB-435-AS2	0/5	3/10

[a] Cells were injected into the MFP region of nude mice as described in "Materials and Methods."
[b] Number of tumors/number of animals.

characterized human breast carcinoma cell lines, namely T47D, MDA-MB-231, MDA-MB-435, BT-549, and SK-Br-3. Of the five, the two cell lines (BT-549 and SK-Br-3), that are nontumorigenic in nude mice do not express galectin-3 (14). The introduction of human rgalectin-3 into the null BT-549 cells resulted in the acquisition of anchorage-independent growth properties and tumorigenicity. The current study was designed to provide more direct evidence that galectin-3 plays a role in the tumorigenicity of breast cancer cells.

As target cells, we chose the well-characterized human breast cancer MDA-MB-435 cells, which are highly tumorigenic and metastatic and overexpress galectin-3 (14, 23) and thus provide an excellent experimental model to study the functional role of galectin-3. Introduction of antisense DNA by transfection of target cells with appropriate expression vectors is a potent approach to inhibit the synthesis of endogenous cellular proteins (24, 25). For this purpose, we constructed an expression vector containing a 707-bp fragment of human galectin-3 genomic DNA in the antisense orientation under the transcriptional control of the cytomegalovirus promoter and the neomycin resistance gene. This antisense DNA was generated from the second intron together with flanking exon sequences of galectin-3 genomic DNA. Therefore, it may hybridize with the primary transcript and/or disrupt transcription. The results presented above indicate that this approach yielded an effective blockage in the synthesis of mature endogenous galectin-3 mRNA and protein (Fig. 1). Using these transfectant cells together with parental MDA-MB-435 cells and a vector control transfectant, we demonstrated the following key findings: (*a*) a decrease of galectin-3 synthesis is associated with change of morphology, acquisition of contact inhibition, reduction of serum independence, and abrogation of anchorage-independent cell growth; (*b*) these effects are not reversed by adding exogenous galectin-3; and (*c*) the inhibition of galectin-3 expression was associated with reduced tumorigenicity in nude mice.

Previously, we have shown that transfection of 3T3 fibroblasts with galectin-3 cDNA results in a morphological change, *i.e.*, loss of contact inhibition, anchorage-independent growth in soft agar, and disruption of structural microfilament organization (26), and that proliferative stimulation of 3T3 fibroblast and infection of normal T lymphocytes that scantily express galectin-3 with T lymphotrophic virus I are associated with increased galectin-3 expression in general and in the nuclei in particular (27, 28) These cumulative data strongly suggested that intracellular galectin-3 may directly contribute to the transformation

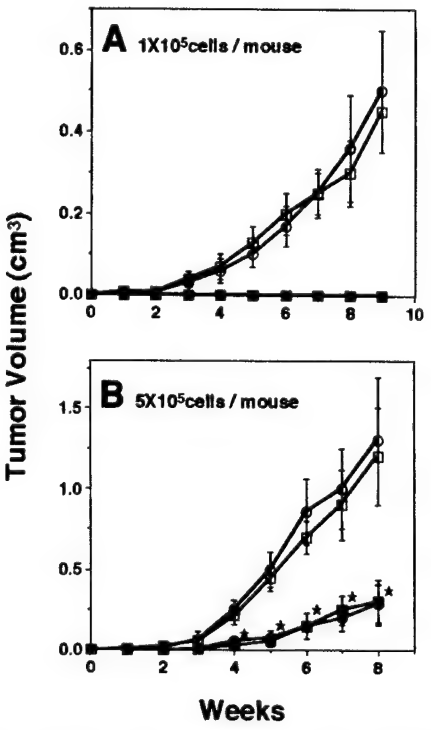

Fig. 5 Tumor growth of MDA-MB-435 (○), MDA-MB-435-CONT (□), MDA-MB-435-AS1(●), and MDA-MB-435-AS2 (■) in nude mice. Either 1×10^5 (*A*) or 5×10^5 (*B*) cells were injected into the MFP region of nude mice (see Table 1 for number of animals per group). Cross-sectional tumor diameters were measured externally, and the approximate tumor volume was calculated as described in "Materials and Methods." *, $P < 0.05$ as compared with parental MDA-MB-435 cells by Mann-Whitney test.

phenotype. However, because we already have shown that galectin-3 is not an oncogene but rather is associated with some aspects of transformation and metastasis (26), it is still unclear in which tumor progression pathway galectin-3 is involved. It is possible that the nuclear exclusion of galectin-3 plays a role in the observed phenomena. Recently, we found that a dissociated expression of cytoplasmic and nuclear galectin-3 during neoplastic progression of the tongue and a reduced expression of galectin-3 from the nucleus were associated with reduced disease-free survival of tongue cancer patients (29). In the prostate, it was suggested that galectin-3 might have antitumor activities when present in the nucleus because cytoplasmic expression is associated with disease progression in a subset of lesions (30). Nuclear and cytoplasmic galectin-3 are likely to be linked with proliferation and differentiation, respectively, in normal epithelium. This assumption may be supported by previous findings that mitogenic stimulation of quiescent fibroblasts results in a prompt increase of nuclear galectin-3 expression (2, 31); nuclear galectin-3 is involved in ribonuclear complexes (32) and has been identified as a factor in pre-mRNA splicing (33). In normal colonic mucosa, cytoplasmic galectin-3 is predominantly observed in the upper areas of the crypt and in the surface epithelium, *i.e.*, terminally differentiated cells (34), and in normal squamous epithelium of the head and neck, distribution of cytoplasmic galectin-3 is confined to the superficial and inter-

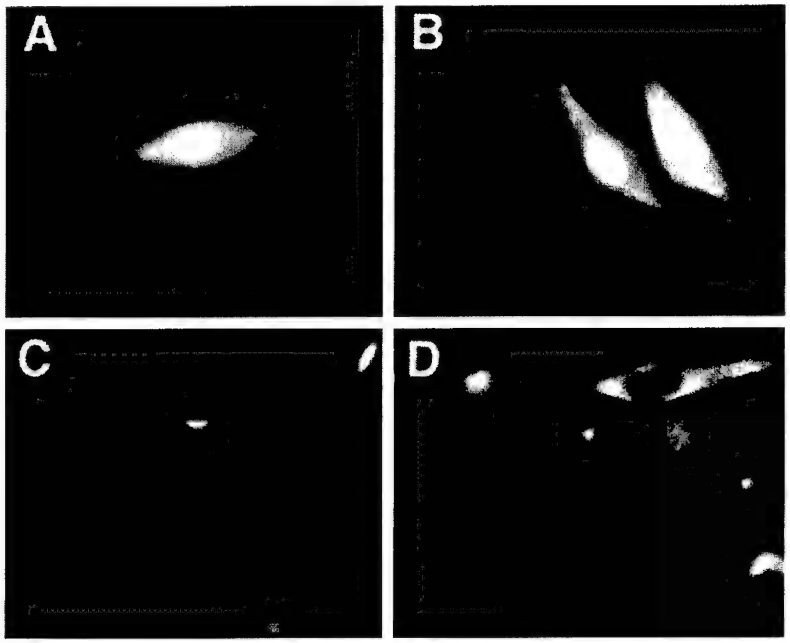

Fig. 6 Immunofluorescence staining of galectin-3 in (*A*) parental MDA-MB-435, (*B*) MDA-MB-435-CONT, (*C*) MDA-MB-435-AS1, and (*D*) MDA-MB-435-AS2 cells. Glass-adherent cells were fixed and stained with anti-galectin-3 antibodies as described in "Materials and Methods."

mediate layers (35). It is also possible that the observed phenomenon is related to the status of the activation of the L1 retrotransposon gene product, which was suggested to contribute together with galectin-3 to the progression of some breast cancers (36). Recently, we have studied the functional role of the unusual leader sequence of galectin-3 and reported that it determines its nuclear localization (37). Transfection of leader sequence galectin-3 deletion mutant into galectin-3-null BT-549 breast cell species failed to convert the cells into tumorigenic ones unlike the wild type, further attesting to the role of galectin-3 nuclear localization in cancer progression.

In conclusion, we have shown that inhibition of galectin-3 in cultured breast cancer cell line is associated with a "normalization" of the cellular phenotype both *in vivo* and *in vitro* and provide direct evidence that galectin-3 plays an important role in breast cancer and may be a target for therapeutic modalities.

ACKNOWLEDGMENTS

We thank Vivian Powell for careful editing of the manuscript.

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Inhibition of Human Cancer Cell Growth and Metastasis in Nude Mice by Oral Intake of Modified Citrus Pectin

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Background: **The role of dietary components in cancer progression and metastasis is an emerging field of clinical importance. Many stages of cancer progression involve carbohydrate-mediated recognition processes. We therefore studied the effects of high pH- and temperature-modified citrus pectin (MCP), a nondigestible, water-soluble polysaccharide fiber derived from citrus fruit that specifically inhibits the carbohydrate-binding protein galectin-3, on tumor growth and metastasis *in vivo* and on galectin-3-mediated functions *in vitro*. *Methods: In vivo* tumor growth, angiogenesis, and metastasis were studied in athymic mice that had been fed with MCP in their drinking water and then injected orthotopically with human breast carcinoma cells (MDA-MB-435) into the mammary fat pad region or with human colon carcinoma cells (LSLiM6) into the cecum. Galectin-3-mediated functions during tumor angiogenesis *in vitro* were studied by assessing the effect of MCP on capillary tube formation by human umbilical vein endothelial cells (HUVECs) in Matrigel. The effects of MCP on galectin-3-induced HUVEC chemotaxis and on HUVEC binding to MDA-MB-435 cells *in vitro* were studied using Boyden chamber and labeling assays, respectively. The data were analyzed by two-sided Student's *t* test or Fisher's protected least-significant-difference test. *Results:* Tumor growth, angiogenesis, and spontaneous metastasis *in vivo* were statistically significantly reduced in mice fed MCP. *In vitro,* MCP inhibited HUVEC morphogenesis (capillary tube formation) in a dose-dependent manner. *In vitro*, MCP inhibited the binding of galectin-3 to HUVECs: At concentrations of 0.1% and 0.25%, MCP inhibited the binding of galectin-3 (10 μg/mL) to HUVECs by 72.1% ($P = .038$) and 95.8% ($P = .025$), respectively, and at a concentration of 0.25% it inhibited the binding of galectin-3 (1 μg/mL) to HUVECs by 100% ($P = .032$). MCP blocked chemotaxis of HUVECs toward galectin-3 in a dose-dependent manner, reducing it by 68% at 0.005% ($P<.001$) and inhibiting it completely at 0.1% ($P<.001$). Finally, MCP also inhibited adhesion of MDA-MB-435 cells, which express galectin-3, to HUVECs in a dose-dependent manner. *Conclusions:* MCP, given orally, inhibits carbohydrate-mediated tumor growth, angiogenesis, and metastasis *in vivo,* presumably via its effects on galectin-3 function. These data stress the importance of dietary carbohydrate compounds as agents for the prevention and/or treatment of cancer. [J Natl Cancer Inst 2002;94:1854–62]**

Carbohydrates have an enormous potential for encoding biologic information. All cells express carbohydrates on their surfaces in the form of glycoproteins, glycolipids, and polysaccharides. Lectins, the carbohydrate-binding proteins, not only distinguish different monosaccharides but also bind specifically to oligosaccharides, detecting subtle differences in complex carbohydrate structures *(1)*. The continuous growth and subsequent metastasis of cancers are dependent on tumor vasculature, and carbohydrate-mediated recognition interactions play a role in angiogenesis *(2)*. Soluble forms of lectins (e.g., E-selectin, vascular cell adhesion molecule-1 [VCAM-1], and P-selectin) can promote endothelial cell migration and morphogenesis after binding to their respective glycoconjugate ligands *(3)*.

The clinical manifestation of an elevated concentration of E-selectin in the sera of cancer patients provides *in vivo* evidence of the importance of these molecules in cancer progression *(4–7)*. However, this premise was challenged by a report *(8)* showing that E- and P-selectin-deficient mice were able to induce normal angiogenesis. Therefore, we previously investigated whether another soluble carbohydrate-binding lectin, i.e., galectin-3, could provide an alternative angiogenic pathway and showed that carbohydrate-dependent galectin-3 binding to endothelial cells induces endothelial cell morphogenesis *in vitro* and angiogenesis *in vivo (9)*. Galectin-3 belongs to the galectin superfamily of proteins, defined by a shared conserved sequence of the carbohydrate-binding domain and affinity to β-galactosides *(10)*.

A notable feature of galectin-3 is its implication in neoplastic transformation and cancer progression. A direct relationship has been shown between galectin-3 levels and the stage of progression of some tumors [for review, *see (11,12)*]. Moreover, experimentally, a monoclonal antibody against galectin-3 strongly inhibits experimental lung metastasis of B16 melanoma and UV-2237 fibrosarcoma cells *(13)*. Synthetic glycoamines Fru-D-Leu and Lac-L-Leu were used as effective inhibitors of spontaneous human breast cancer metastasis in nude mice *(14)*, and D-galactose and arabinogalactan substantially inhibited the formation of experimental liver metastasis of L-1 sarcoma cells *(15)*. More recently, it was reported that anti-galectin-3 antibody and lactose inhibit liver metastasis by adenocarcinoma cell lines XK4A3 and RPMI4788 *(16)*. These studies suggest the potential for carbohydrate-mediated cancer therapy.

Pectin is a highly complex branched polysaccharide fiber rich in galactoside residues and present in all plant cell walls. Initially it was reported to bind to the carcinogen 1,2-dimethylhydrazine (DMH) with an increasing efficiency as pH was raised

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See "Notes" following "References."

from acidic to alkaline *(17)*. In its native form, citrus pectin (CP) has a limited solubility in water and is unable to interact with galectin-3, but in its modified form (MCP) after hydrolysis to form a smaller linear water-soluble fiber, it acts as a ligand for galectin-3 *(18–20)*. Injection of MCP-treated mice with melanoma B16-F1 cells resulted in a statistically significant reduction in lung colonization *(19)*. Furthermore, oral administration of MCP to male Copenhagen rats injected with the prostate cancer cell line MAT-LyLu reduced spontaneous lung colonization in a dose-dependent fashion *(18)*, suggesting that MCP interferes with galectin-3-dependent tumor embolization in the circulation, leading to reduced metastasis *(18,19)*. A reduction in the growth of colon tumors implanted in mice after oral administration of MCP has been demonstrated *(21)*. Hsieh and Wu *(22)* have recently reported that MCP treatment of human prostatic JCA-1 cells reduced cell growth and DNA synthesis, which was associated with reduced expression of cyclin B, nm23, p34, and cdc2.

The role of dietary nondigestible, water-insoluble carbohydrate fibers in the etiology of various human cancers is of considerable interest, because their use as chemopreventive agents has important implications for cancer prevention. Data from our laboratories *(18–20)* and others *(22–26)* have shown that carbohydrate-supplemented diets can inhibit tumor growth and metastasis in experimental murine tumor systems. Here, we studied the effect of MCP on galectin-3-mediated functions *in vitro* and on angiogenesis, tumor growth, and metastasis *in vivo* in athymic mice.

Materials and Methods

Cell Lines and Culture

Human umbilical vein endothelial cells (HUVECs) were purchased from the American Type Culture Collection (ATCC, Manassas, VA). The metastatic human breast cancer cell line MDA-MB-435 was a gift from Dr. Eric W. Thompson (St. Vincent's Institute of Medical Research and University of Melbourne, Melbourne, Australia). LSLiM6 is a well-characterized metastatic colonic adenocarcinoma cell line derived from low metastatic LS174T *(27,28)*. HUVECs were cultured in Ham's F12K medium (Irvine Scientific, Irvine, CA) supplemented with 100 μg/mL heparin (Sigma Chemical Co., St. Louis, MO), 50 μg/mL endothelial cell growth supplement (Collaborative Biomedical Products, Bedford, MA), and 10% fetal bovine serum (FBS; Summit Biotechnology, Fort Collins, CO). MDA-MB-435 and LSLiM6 cells were maintained in Dulbecco's Minimal Essential Medium (Invitrogen Corporation, Carlsbad, CA) containing 10% heat-inactivated fetal calf serum (FCS), essential and nonessential amino acids (Invitrogen Corporation), vitamins, and antibiotics (Mediatech, Inc., Herndon, VA). The cells were maintained in a humidified chamber with 95% air and 5% CO_2 at 37 °C. The cells were grown to confluence and detached from the monolayer with 0.25% trypsin with 2 m*M* EDTA. The use of the cell lines was approved by the Human Investigations Committee, Wayne State University (Detroit, MI).

For collection of conditioned media, the cells were plated to confluence in a 60-mm dish. After 24 hours the medium was removed, and the cells were washed thoroughly with phosphate-buffered saline (PBS) and allowed to grow in serum-free medium. The medium was collected after 24 hours, concentrated 20-fold by centrifugation through Ultrafree-MC centrifugal filter units (Millipore, Bedford, MA) with a 10 K molecular weight cut-off, and analyzed for the presence of galectin-3 by Western blot analysis.

Preparation of Recombinant Galectin-3 and Modified CP

Recombinant human galectin-3 was expressed in *Escherichia coli* and isolated by affinity chromatography using an asialofetuin column as previously described *(29)*. CP was purchased from Sigma Chemical Co.; pH and temperature modification of pectin was performed as described *(19)*. Briefly, CP was solubilized as a 1.5% solution in distilled water, and its pH was increased to 10.0 with NaOH (3 N) for 1 hour at 50–60 °C. The solution was then cooled to room temperature while its pH was adjusted to 3.0 with 3 N HCl and stored overnight. Samples were precipitated the next day with 95% ethanol and incubated at –20 °C for 2 hours, filtered, washed with acetone, and dried on Whatman filters. For oral feeding of the nude mice, a 1% solution of MCP was prepared in autoclaved water, its pH was adjusted to approximately 7.0, and the solution (500 mL) was sterilized by γ-irradiation using a Mark 1–68 irradiator (J. L. Shepherd & Associates, Glendale, CA) at 552 Rads/minute for 45 minutes.

Composition Analysis of CP and MCP

Composition analysis was performed at the Complex Carbohydrate Research Center, University of Georgia (Athens). The samples were hydrolyzed using freshly prepared 1 *M* HCl in 3% methanol for 16 hours at 80 °C. The released sugars were dried down and N-acetylated using methanol and acetic anhydride for 15 minutes at 45 °C. The acetylated sample was trimethyl sialylated with Tri-Sil reagent (Pierce, Rockford, IL) and resolved on a 30-m DB-1 column (J&W Scientific, Folsom, CA) on a 5985 GC-MS system (Hewlett-Packard, Palo Alto, CA) using myoinositol as an internal standard.

Tumor Growth and Metastasis

NCR nu/nu mice were injected in the mammary fat pad region with 7.5×10^5 MDA-MB-435 cells. The site, time of inoculation, and autopsy were as described by Price et al. *(30)*. Two groups of 10 mice each were given 1% (w/v) MCP (pH ≈7.0) in drinking water starting 1 week prior to the injections of tumor cells. Two control groups of 10 mice each were maintained on regular autoclaved water. For one group each of MCP-fed and control mice, the tumors were measured twice a week for 7 weeks, and the volumes were calculated by formula (length × width_1 × width_2 × 0.5). After 51 days, the mice were anesthetized (with ketamine [70 mg/kg body weight] and xylazine [7.5 mg/kg body weight]), and the primary tumors were surgically removed because some of them were larger than 1.5 cm. The mice continued to receive water or MCP solution for 8 more weeks, after which they were killed by cervical dislocation. The lungs were removed, fixed with Bouin's fixative, and examined visually as well as microscopically for the formation of tumor cell colonies.

The other groups of control and MCP-fed mice were killed at day 33. The tumors were removed, weighed, fixed with 10% formalin in PBS, and processed for immunohistochemical staining of blood vessels. The tumors were removed at 33 days, because some tumors became necrotic at later stages.

The ability of MCP to inhibit spontaneous metastasis of human colon cancer cells from the cecum to the liver was tested as previously described *(28)*. We selected the colon adenocarcinoma cell line LSLiM6, because these cells exhibit a high liver metastasis during cecal growth and high liver-colonizing ability after splenic portal injection in nude mice *(27)*. These cells also produce high levels of intracellular and cell surface galectin-3 *(28)*. A group of 10 pathogen-free nude mice was fed 1% MCP in their drinking water for 1 week, after which they were anesthetized with methoxyflurane by inhalation, the cecum was exteriorized, and 5×10^6 viable LSLiM6 cells in 0.1 mL were injected into the cecal wall. The cecum was replaced *in situ*, and the abdomen was closed with stainless steel clips. After 6 weeks, the animals were killed, and the cecum, abdominal tumor mass, and liver were removed. The number of animals with macroscopic liver nodules was determined and compared with that of control animals given plain water. All procedures were carried out in accordance with the guidelines provided by the Animal Investigation Committee at Wayne State University. All mice were checked daily, and no variations in body weight or behavior among the control and treated mice groups were observed.

Immunohistochemical Analysis to Visualize Blood Capillary Vessels in Primary Tumors

To visualize the blood vessels in the primary tumors removed from control and MCP-fed mice, the sections were stained with alpha smooth muscle actin, which stains the smooth muscle cells of the vessels. Immunohistochemistry was performed using a modification of the avidin–biotin–peroxidase complex technique. Briefly, 4-μm tissue sections were deparaffinized, rehydrated, and placed in a 3% hydrogen peroxide solution to inhibit endogenous peroxidase. The tissue sections were treated with 0.1% trypsin and 0.1% $CaCl_2$ for 30 minutes at 37 °C to expose the antigenic sites masked by formalin fixation, blocked for 1 hour with 3% normal goat serum (Sigma Chemical Co.), and subsequently incubated overnight with monoclonal mouse anti-human alpha smooth muscle actin (DAKO, Carpinteria, CA) at a dilution of 1 : 100. The sections were then treated with biotinylated secondary antibody (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA) for 30 minutes at room temperature, followed by avidin-biotinylated horseradish peroxidase (HRP) complex reagent (according to the manufacturer's instructions) for 30 minutes and diaminobenzidine (Sigma Chemical Co.) for 1 minute. Counterstaining was performed with hematoxylin.

Capillary Tube Formation by HUVECs

Capillary tube formation by HUVECs was assayed on Matrigel (Collaborative Biomedical Products, Bedford, MA) as described earlier *(9)*. To prepare a gel, 200 μL of Matrigel thawed on ice was added to each chamber of an eight-chamber slide. The air bubbles were carefully removed, and the slide was transferred to a 37 °C incubator for 15 minutes. After gelation, 5×10^4 endothelial cells, separated from monolayers with trypsin treatment, were plated onto the gel in 200 μL of medium. In some chambers, MCP or CP was added to the cells at the time of incubation. The tube formation was observed after 16 hours.

Chemotaxis of HUVECs in Response to Galectin-3

Galectin-3-induced chemotactic response of endothelial cells was analyzed using a Boyden chamber. Briefly, 30 μL of serum-free F12K medium containing 10 μg/mL galectin-3 in the presence or absence of various concentrations of MCP was added to the lower chamber as a chemoattractant. HUVECs (5×10^4) were added to the upper chamber. The two chambers were separated by polycarbonate filters (8-μm pore size) and incubated at 37 °C. After 5 hours, the cells attached to the lower surface of the filter were fixed and stained using the Protocol Hema 3 Stain set (Fisher Scientific Company, Pittsburgh, PA). Cells in a total of 10 fields from each chamber were counted under a microscope, and the average number of cells per field was plotted. Each assay was carried out in quadruplicate. To investigate the specificity of chemotaxis inhibition by MCP, a comparative evaluation was performed using fibronectin and basic fibroblast growth factor (bFGF)-induced chemotaxis.

Biotinylation of Recombinant Galectin-3

Recombinant galectin-3 was isolated from transformed bacteria and purified as described previously *(29)*. The protein was biotinylated using an EZ-Link Sulfo-NHS-Biotinylation Kit (Pierce) according to the manufacturer's instructions. Briefly, the protein solution was concentrated to 2 mg/mL in PBS and mixed into 30 μL of sulfo-NHS-biotin (2 mg/100 μL H_2O) to get a molar ratio of 1 : 20. Biotinylation was achieved by incubating the mixture on ice for 2 hours. Excess salt was removed by passing the protein solution through the desalting column. The fractions were collected, and the protein content of each fraction was determined.

Galectin-3-HUVEC Binding Assay

HUVECs were seeded at a density of 1×10^4 cells/well in a 96-well plate. After 24 hours, the cells were washed four times with PBS and incubated with various concentrations of MCP in 100 μL of serum-free F12K medium for 15 minutes at 37 °C. After incubation, various concentrations of biotinylated recombinant galectin-3 were added to the wells and incubated for 2 hours at 37 °C. The wells were washed carefully three times with PBS. Next, 100 μL of a 1 : 1000 dilution of HRP-conjugated streptavidin was added to the wells and incubated at room temperature for 30 minutes. Unbound proteins were removed by washing three times with PBS. Color development was obtained by using 100 μL of citrate buffer mixed with ABTS (2,2 azino-di[3-ethylbenzthiazoline]sulfonic acid) and H_2O_2 and measured by an enzyme-linked immunosorbent assay (ELISA) plate reader (Molecular Devices, Sunnyvale, CA) at a wavelength of 405 nm.

Indirect Immunofluorescence Assay to Detect Galectin-3 on Tumor Cells

MDA-MB-435 cells were trypsinized and seeded in a four-chamber slide at a density of 50 000 cells/chamber. After 24 hours, the cells were fixed with 3.4% paraformaldehyde for 15 minutes at room temperature and washed four times with PBS containing 1% bovine serum albumin (BSA). The cells were then blocked for 30 minutes with 1% BSA in PBS followed by incubation with primary antibody (TIB-166 anti-galectin-3 monoclonal antibody made in rat; ATCC) at a 1 : 1 dilution with 1% BSA in PBS at 4 °C for 1 hour. Subsequently, the cells were washed three times with 0.1% BSA in PBS and incubated with fluorescein isothiocyanate (FITC)-labeled goat anti-rat immunoglobulin G (IgG; Zymed, San Francisco, CA) at a 1 : 50 dilution for 30 minutes. The primary antibody was omitted

in controls. The chambers were peeled off the slide, and the cells were mounted in gelvatol (13% w/v polyvinyl alcohol-2000, 0.6× PBS, and 30% glycerol) and observed under a fluorescence microscope (Olympus, Tokyo, Japan) for the presence of galectin-3.

Western Blot Analysis for Galectin-3

To study the expression of galectin-3, HUVECs or MDA-MB-435 cells were trypsinized and mixed with trypan blue. The viable cells were counted by a hemacytometer, and cells were suspended in a sample buffer (0.76% Tris, 10% glycerol, 1% sodium dodecyl sulfate [SDS], 1% 2-mercaptoethanol, 1% bromophenol blue) at 5000 cells/μL. To study the secretion of galectin-3, the conditioned media were collected and concentrated as described in the "Cell Lines and Culture" section. Equal amounts of protein (50 μg) or lysed cells (1×10^5) were loaded in each lane. The proteins were separated on a 12.5% polyacrylamide separating gel and a 3.5% stacking gel and electroblotted to polyvinylpyrrolidine fluoride (PVDF) membranes (MSI, Westborough, MA). Nonspecific binding was blocked in 5% nonfat dry milk in PBS for 1 hour, followed by incubation with the first antibody (rat monoclonal anti-galectin-3 or rabbit polyclonal anti-galectin-3 antibody) for 1 hour at room temperature. Subsequently, the membranes were washed five times with a blocking solution containing 0.1% Tween 20 and incubated with secondary antibody (HRP-conjugated rabbit anti-rat IgG or goat anti-rabbit IgG, respectively; Zymed) for 1 hour. After washing as before, they were processed for enhanced chemiluminescence using ECL western blotting detection reagents (Amersham, Piscataway, NJ) to locate the galectin-3 protein, according to the manufacturer's instructions.

Tumor Cell–HUVEC Adhesion Assay

MDA-MB-435 cells were suspended at a concentration of 3×10^6 cells/mL in serum-free medium containing 1% BSA and radiolabeled with 5 μCi of $Na^{51}CrO_4$ (Dupont NEN Research Products, Boston, MA) for 2 hours at 37 °C. At the end of the incubation, the cell suspensions were washed extensively and plated in quadruplicate in 16-mm Costar culture dishes (Corning Costar, Cambridge, MA) containing HUVEC monolayers in the presence or absence of different final concentrations (0.01%, 0.05%, 0.1%, or 0.25%) of MCP. After 2 hours, the cells were washed gently and thoroughly with PBS, and the attached cells were lysed with 0.1 N NaOH (30 minutes, 37 °C). To determine the percentage of adhesion of MDA-MB-435 cells to HUVECs, the cell-associated radioactivity was determined in a Packard Auto Gamma Counter (model 5650; Packard Biosciences Co./Perkin Elmer, Downer's Grove, IL). The adhesion of tumor cells to HUVECs in control experiments without MCP was given the value of 100%; percent adhesion in the presence of MCP was calculated accordingly.

Statistical Analysis

Tumor growth, chemotaxis, angiogenesis *in vivo,* and binding were the primary outcomes measured. The data were provided as means of either two or three experiments with 95% confidence intervals (CIs). (The experiments conducted to measure growth of the tumors were repeated twice with multiple animals.) We used Student's *t* test or Fisher's protected least-significant-difference (PLSD) test from StatView software (Abacus Concepts, Inc., Berkeley, CA) to analyze the statistical significance of the results. All statistical tests were two-sided, and *P* values less than .05 were considered statistically significant.

Results

To test the ability of MCP to inhibit primary tumor growth and metastasis, 7.5×10^5 MDA-MB-435 human breast carcinoma cells were injected into the mammary fat pad of NCR nu/nu mice previously fed for a week with 1% MCP in their drinking water. The pH of the solution was adjusted to 7.0 to neutralize the acidic taste of MCP. The mice were fed continuously with MCP throughout the duration of the experiments. The addition of MCP to the drinking water did not affect the overall tumorigenic efficiency. However, a statistically significant reduction in tumor growth rate was observed in mice fed with MCP compared with that in mice from the control group ($P = .050$, Student's *t* test; Fig. 1). The tumors in control mice reached 1.5 cm at 7 weeks after tumor inoculation, forcing us to terminate the analysis in all mice. The average tumor volume for control mice was 552 ± 14 mm^3 (95% CI = 540 to 564 mm^3) versus 165 ± 48 mm^3 (95% CI = 128 to 201 mm^3) for MCP-fed mice. The difference in tumor volumes between the control group and the treated group was 387 mm^3 (95% CI = 363 to 412 mm^3). At the termination of the experiments, after 15 weeks, the experimental mice were killed, autopsied, and examined for tumor metastases. Three mice (one control mouse and two MCP-fed mice) died during or immediately after surgery; therefore, the metastasis was not analyzed in all 10 mice in each group. The number of mice with lung metastases was statistically significantly smaller in the MCP-fed group than in the control group (zero of eight mice versus six of nine mice, respectively). Representative lungs from each group are depicted in Fig. 2. Daily water intake was similar in all groups. The mice did not show any dislike for MCP. Animal body weight and overall behavior were similar in the control group and the treated group.

To analyze whether MCP would inhibit the growth of other tumor types, we also studied the colonic growth of human colon carcinoma cells and spontaneous metastasis in MCP-fed nude mice. Previously, we had shown that galectin-3 plays a role in

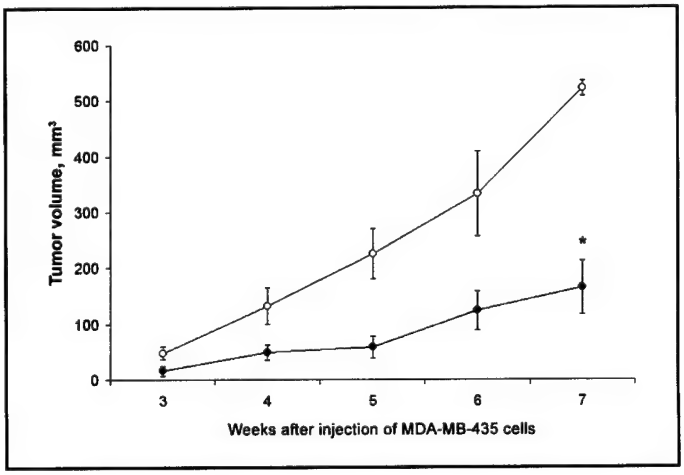

Fig. 1. Tumor growth in modified citrus pectin (MCP)-fed mice. MDA-MB-435 cells were injected into the mammary fat pad region of nude mice, and tumor volumes were measured twice a week. At the end of 51 days, the tumor volumes were calculated from 10 mice. Larger tumors were observed in the control mice (**open circles**) than in the MCP-fed mice (**closed circles**). The **error bars** represent 95% confidence intervals. *, $P = .050$.

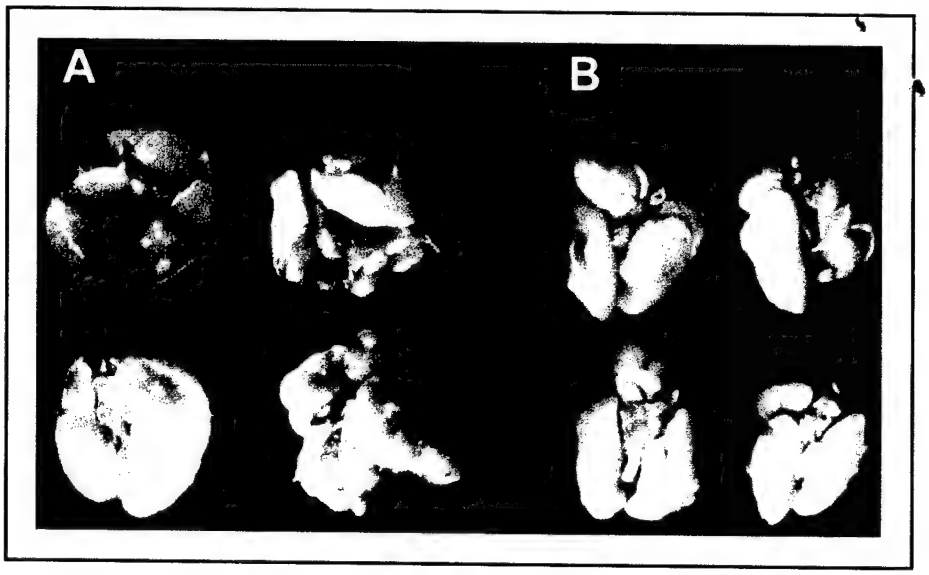

Fig. 2. Lung metastasis of MDA-MB-435 cells in modified citrus pectin (MCP)-fed mice. Mice were injected with 0.75×10^6 cells in the mammary fat pad region. Some mice were fed water alone (**A**) and others received 1% MCP in their drinking water (**B**). Tumors were removed after 7 weeks, and after 8 more weeks, the mice were killed and the presence or absence of lung metastasis (nodules on lung surface) was recorded.

liver colonization of human colon carcinoma cells after splenic-portal or cecal growth *(27,28)*. We therefore examined whether MCP would affect the dissemination of these cells from the cecum to the liver. Five million LSLiM6 cells were surgically implanted into the cecum of nude mice (10 each of control and MCP-fed mice), and 6 weeks later, after continuous MCP feeding, the mice were killed, tumors were excised and weighed, and the incidence of metastasis was recorded. The average weights of the primary tumors in the control and 1% (w/v) MCP-fed mice were 1.16 g (95% CI = 1.13 to 1.19 g) and 0.65 g (95% CI = 0.37 to 0.93 g), respectively. The intra-abdominal tumor weights in the control and the MCP-fed mice were 2.0 g (95% CI = 1.94 to 2.06 g) and 0.88 g (95% CI = 0.37 to 0.93 g), respectively. The difference in primary tumor weight between the control group and the treated group was 0.51 g (95% CI = 0.40 to 0.82 g). The difference in intra-abdominal tumor weights between the control group and the MCP-fed group was 1.12 g (95% CI = 1.01 to 1.69 g). Metastases to lymph nodes and to the liver were present in 100% (nine of nine) and 60% (six of 10) in control mice, respectively, versus in 25% (two of eight) and 0% (zero of nine) in the MCP-fed mice. Similar results were observed in repeat experiments. Daily water intake was similar in all groups. Animal body weight and overall behavior were unchanged between the control group and the treated group.

We have shown previously that galectin-3 mediates endothelial cell morphogenesis *in vitro* and angiogenesis *in vivo* in a carbohydrate-dependent manner *(9)*. Thus, we investigated whether the inhibitory effects of MCP on tumor growth were associated with reduced angiogenesis. Primary MDA-MB-435 tumors growing in the mammary fat pad of water- or MCP-fed nude mice (five mice per group) were excised, fixed, and stained for the presence of blood vessels. The tumors from MCP-fed mice had one-third the number of vessels per unit area as the tumors in the control mice (Fig. 3). Three tumors from each group were sectioned, and one field per slide was counted from three slides per tumor for the blood vessels. The average number of blood vessels (and 95% CIs) for control and MCP-fed mice, respectively, were 15.0 (95% CI = 12.9 to 17.2) and 4.9 (95% CI = 3.0 to 6.7). To directly test the effect of MCP on endothelial cell morphogenesis, an *in vitro* capillary tube formation assay was performed. A thin layer of Matrigel was formed in each chamber of an eight-chamber slide by incubation at 37 °C for 15 minutes. Fifty thousand HUVECs were then plated in each chamber, along with varying concentrations of MCP or CP. A dose-dependent inhibition of the ability of the cell to form a capillary network on Matrigel in the presence of MCP (Fig. 4, B–D) was observed as compared with control PBS (Fig. 4, A) and intact CP controls (Fig. 4, E and F).

Chemotaxis is an integral part of angiogenesis, invasion, and metastasis *(31)*. Like bFGF, galectin-3 induces a chemotactic response in HUVECs *(9)*. To determine whether MCP would inhibit galectin-3-induced chemotaxis of endothelial cells, we performed Boyden chamber chemotaxis assays. As a chemoattractant, galectin-3 (10 μg/mL) in serum-free medium containing various concentrations of MCP was placed in the lower chamber, and HUVECs (5×10^4 cells) were loaded into the upper chambers. After 5 hours of incubation at 37 °C, the cells that migrated toward the chemoattractant were fixed, stained, and counted under a phase-contrast microscope (Fig. 5, A). MCP statistically significantly inhibited the chemotactic response of galectin-3 in a dose-dependent manner. At a concentration of 0.005% (w/v), chemotaxis was reduced by 68%. At a concentration of 0.1% (w/v), there was a complete inhibition of chemotaxis, with the same number of migratory cells as in the negative control. Next, we investigated whether the effect of MCP is specific to the chemotactic response to galectin-3 by analyzing fibronectin- and bFGF-induced chemotaxis in the presence of MCP. MCP had a small inhibitory effect (at a concentration of 0.05%) on fibronectin-induced migration (26% inhibition) and strongly reduced bFGF-induced chemotaxis (by 34% and 86% at concentrations of 0.01% and 0.05%, respectively) (Fig. 5, B). Calculated *P* values using Fisher's PLSD test for galectin-3 were .016 with 0.001% MCP and less than .001 with 0.005%, 0.01%, and 0.1% MCP; for fibronectin, the *P* values were .122 and .007 at 0.01% MCP and 0.05% MCP, respectively; for bFGF, the *P* values were .015 and less than .001 at 0.01% MCP and 0.05% MCP, respectively.

We have previously demonstrated that galectin-3 binds to endothelial cell surface high- and low-affinity receptors *(9)* and that this binding specifically initiates endothelial cell capillary tube formation. To establish whether MCP inhibits this binding, 1×10^4 HUVECs were plated in a 96-well plate and incubated

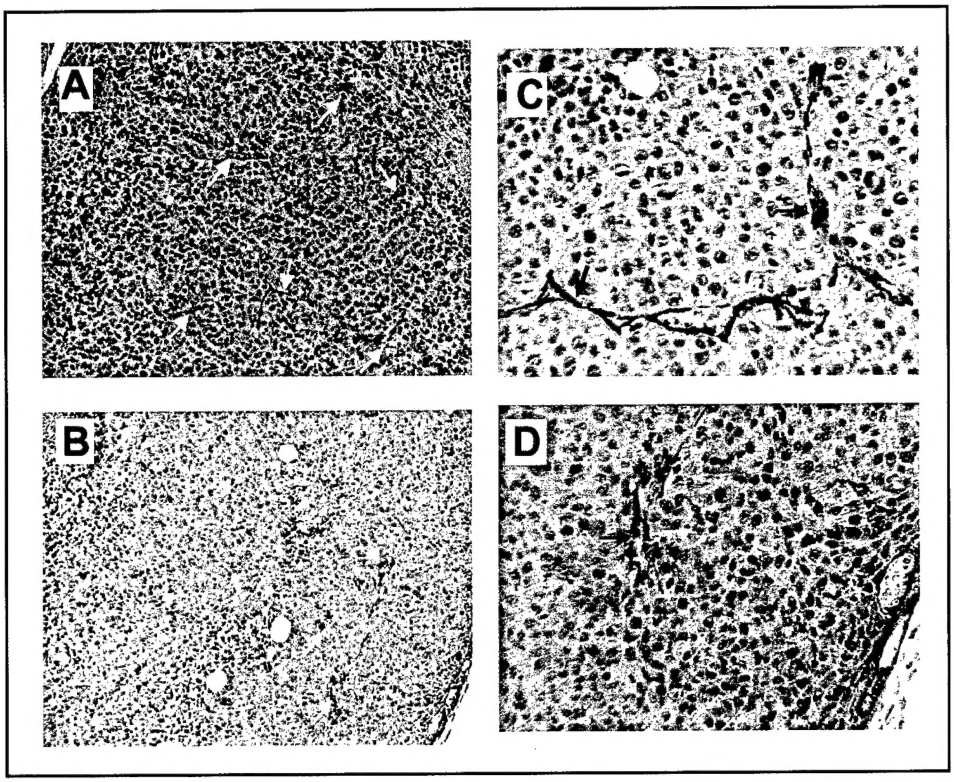

Fig. 3. *In vivo* angiogenesis in modified citrus pectin (MCP)-fed mice. Nude mice were injected with breast cancer cells. Tumors were removed after 33 days, fixed, embedded in paraffin, sectioned, and stained for the presence of blood vessels using antibody against smooth muscle actin. Three tumors from each group were sectioned, and three slides per tumor were studied. **A**) Control, ×100; **B**) MCP-fed, ×100; **C**) control, ×250; **D**) MCP-fed, ×250.

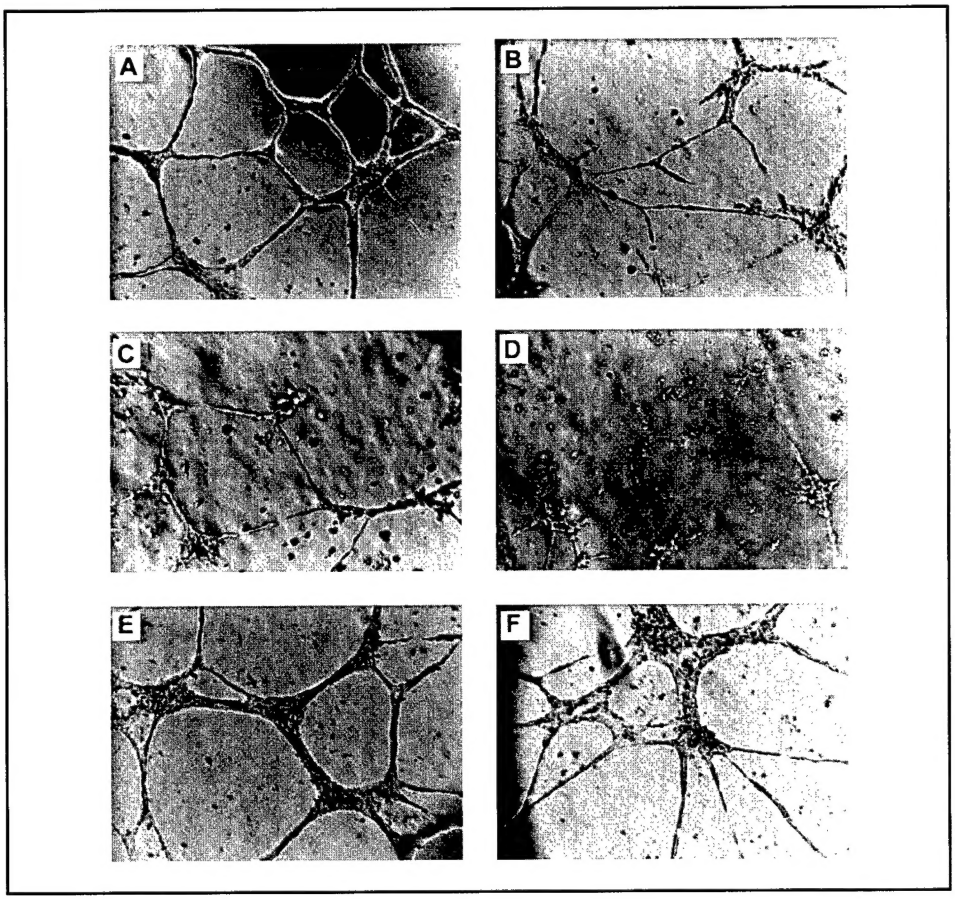

Fig. 4. Inhibition of *in vitro* capillary tube formation. Human umbilical vein endothelial cells were plated on gelled Matrigel (200 μL/chamber) at a density of 50 000 cells per chamber in the absence (**A**) and presence of 0.01% (**B**), 0.05% (**C**), or 0.1% (**D**) modified citrus pectin (MCP) or 0.05% (**E**) or 0.1% (**F**) citrus pectin (CP). The ability to form tubes was inhibited in the presence of MCP compared with control (**A**) or CP (**E** and **F**). The experiment was repeated three times, and representative pictures are shown.

for 15 minutes at 37 °C with various concentrations of MCP. Biotinylated galectin-3 (1 μg/mL and 10 μg/mL) was then added, and after 2 hours of incubation at 37 °C, the cells were thoroughly washed and binding efficiency was determined by color development with ABTS and H_2O_2. The results (Fig. 6) show that galectin-3 bound to HUVECs and that MCP specifically inhibited this binding. Similar experiments were performed with CP, lactose, and sucrose; inhibition was seen with lactose

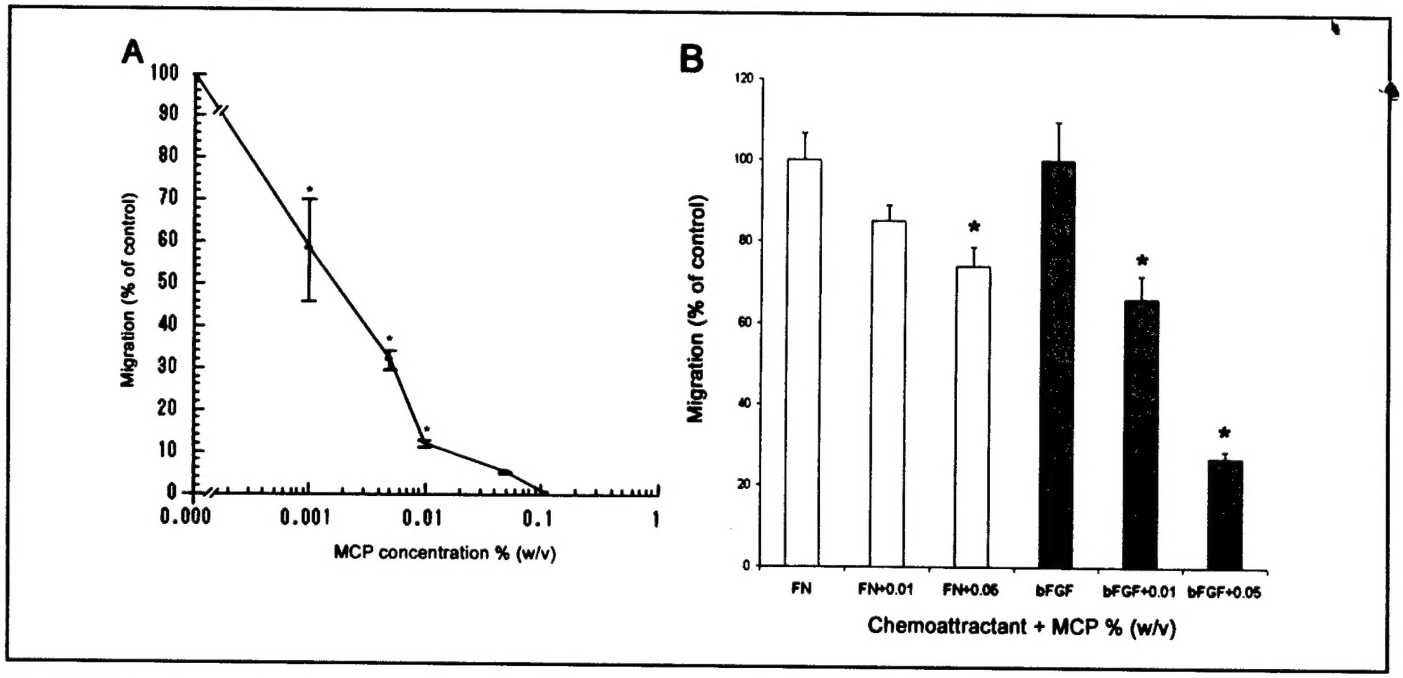

Fig. 5. Inhibition of chemotaxis. **A**) Galectin-3 was added in the lower chamber with different concentrations of modified citrus pectin (MCP). In the upper chamber, 5×10^4 human umbilical vein endothelial cells were loaded. The two chambers were separated by a polycarbonate filter of 8-μm pore size and incubated at 37 °C for 5 hours. The number of cells migrating to the lower side of the filter was calculated. Each point represents an average of eight readings. **B**) Same experiment performed using fibronectin (FN) with or without MCP (**open bars**) and basic fibroblast growth factor (bFGF) with or without MCP (**gray bars**). **Error bars** represent 95% confidence intervals. *, *P* values using Fisher's protected least-significant-difference test in **A:** .016 with 0.001% MCP and less than .001 with both 0.005% and 0.01% MCP; in **B:** .007 and .122 at 0.05% and 0.01% MCP, respectively, when used with fibronectin; and .015 and less than .001 at 0.01% and 0.05% MCP, respectively, when used with bFGF.

and MCP only and not with sucrose or CP (data not shown). The binding of 10 μg/mL galectin-3 to HUVECs was inhibited by 72.1% and 95.8%, respectively, with MCP concentrations of 0.1% and 0.25%, and binding of 1 μg/mL galectin-3 to HUVECs was inhibited by 100% in the presence of 0.25% MCP. (*P* values using Fisher's PLSD test were .045 and .032 at MCP concentrations of 0.1% and 0.25% for a 1 μg/mL galectin-3 concentration and .038 and .025 at MCP concentrations of 0.1% and 0.25% for a 10 μg/mL galectin-3 concentration.) Western blot and direct immunofluorescence analyses showed that MDA-MB-435 cells express galectin-3 on the cell surface and in the cytoplasm and secrete it (Fig. 7). There was a progressive inhibition (33%, 58.4%, 66.5%, and 83.4%) of the binding ability (adhesion) of these tumor cells to the HUVECs by increasing doses of MCP, i.e., 0.01%, 0.05%, 0.1%, and 0.25%, respectively (Fig. 8). *P* values, as calculated by Fisher's PLSD test, were less than .001 at 0.05%, 0.1%, and 0.25% MCP and .003 at 0.01% MCP. Thus, inhibition of tumor cell–endothelial cell interaction by MCP may affect adhesive interactions that play a role in invasion and metastasis.

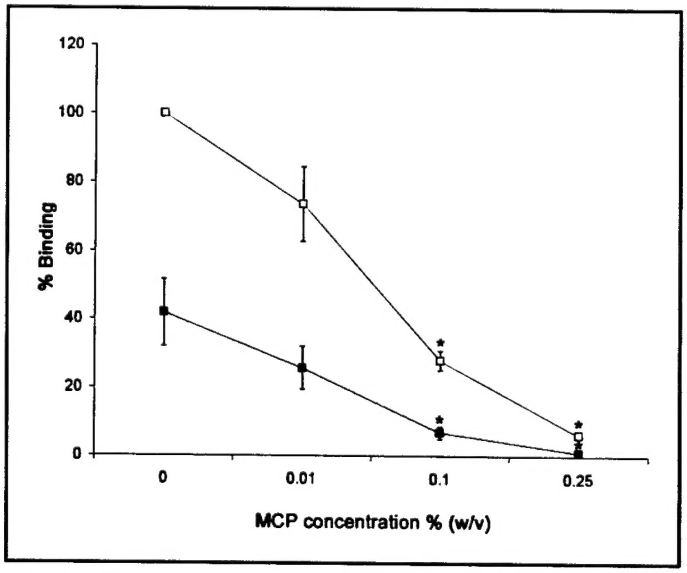

Fig. 6. Binding of recombinant galectin-3 to human umbilical vein endothelial cells (HUVECs) in the presence of modified citrus pectin (MCP). Recombinant galectin-3 was biotinylated using the EZ-Link Sulfo-NHS-Biotinylation kit (Pierce). Endothelial cells were incubated with 1 μg (**closed squares**) or 10 μg/mL (**open squares**) galectin-3 in the presence of 0.01%, 0.1%, or 0.25% MCP. Binding was determined by color development as described in the "Materials and Methods" section. Optical density of cells incubated with 10 μg of galectin-3 was arbitrarily given a value of 100% binding. Other values were calculated accordingly. Each point represents a mean of three readings. **Error bars** represent 95% confidence intervals. *, *P* values using Fisher's protected least-significant-difference test were .045 and .032 at MCP concentrations of 0.1% and 0.25%, respectively, for 1 μg of galectin-3 and .038 and .025 at MCP concentrations of 0.1% and 0.25%, respectively, for 10 μg/mL galectin-3.

Discussion

The use of dietary components having protective and/or preventive effects on cancer progression and metastasis is an important emerging field of research. Identifying new food supplements and understanding their mechanisms of action are some of the main challenges in using functional foods as a cancer therapeutics modality.

It has been previously shown that pectin hydrolysate, whether administered orally *(18)* or intravenously *(19)*, reduces both the spontaneous and experimental lung colonization of tumor cells. CP in the form of water-insoluble fibers also may reduce the

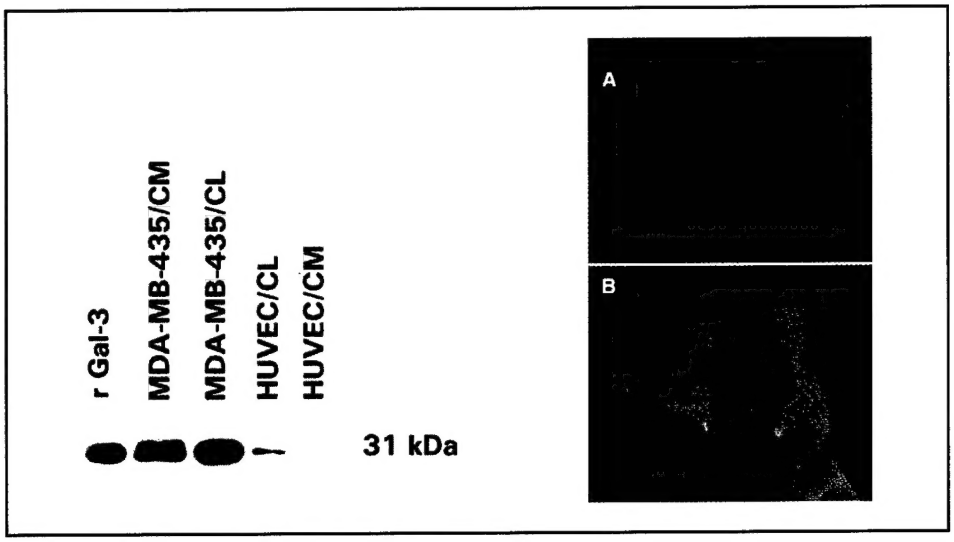

Fig. 7. Left panel: Western blot analysis of conditioned media (CM) and total cell lysates (CL) of MDA-MB-435 and human umbilical vein endothelial cells (HUVECs). For comparison, 100 ng of recombinant galectin-3 (r-gal-3) was loaded into one lane. **Right panel:** Indirect immunofluorescence of MDA-MB-435 cells for surface expression of galectin-3. **A**) Negative control, in which the primary antibody was omitted. **B**) Stained cells expressing galectin-3.

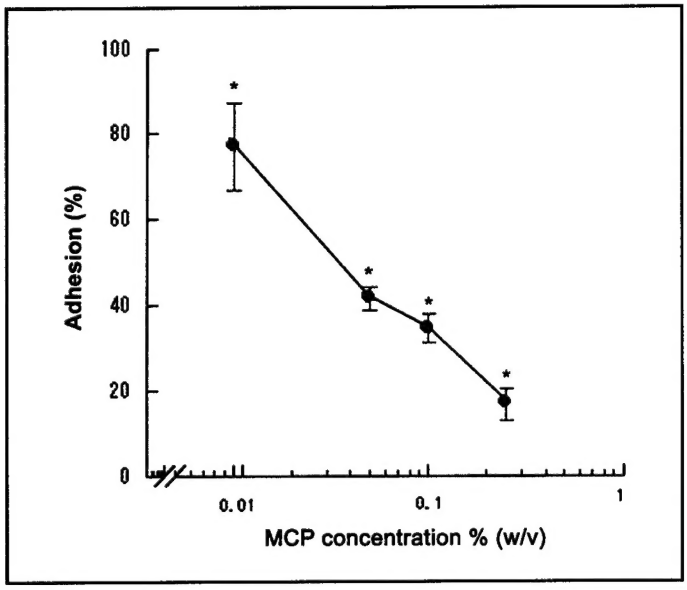

Fig. 8. Adhesion of tumor cells to human umbilical vein endothelial cells (HUVECs). MDA-MB-435 breast cancer cells were labeled with $Na^{51}CrO_4$ and incubated with HUVECs in the presence of various concentrations of modified citrus pectin (MCP). After 2 hours, the cells were washed, lysed, and counted by scintillation counter. Controls were given a value of 100%, and the other values were calculated accordingly. Each value represents a mean of three readings. **Error bars** represent 95% confidence intervals. *, $P<.001$.

incidence of chemically induced colon cancer *(17),* presumably by promoting *Bifidobacteria (26).* Rats fed on a 15% CP-enriched diet showed a higher apoptotic index in their colon *(23–25).* Reduced cell growth and corresponding [^{3}H]thymidine incorporation into DNA was reported when human prostatic JCA-1 cells were grown in media containing MCP *(22).* Pectins have also been found to exhibit anti-mutagenic activity against nitroaromatic compounds *(32).* Daily oral administration of MCP reduced the growth of implanted colon-25 tumors in BALB/c mice, and dietary pectin reduced the growth of intramuscularly transplanted mouse tumors from tumor cell lines TLT and EMT6 *(33).* The results presented in the current study are the first report showing an inhibition of tumor growth and metastasis of orthotopically grown breast and colon cancer cells by a soluble, orally ingested dietary carbohydrate fiber.

The data indicate that MCP might reduce mammary and colonic tumor growth and metastasis by inhibiting angiogenesis. In mammary carcinoma cells growing in the mammary fat pad of nude mice, we observed a 70.2% reduction in the mean tumor volume by 7 weeks following the oral intake of MCP (Fig. 1). This was associated with a 66% reduction in blood vessels and a complete inhibition of metastasis to the lungs (Fig. 3). Similarly, there was less tumor burden and metastasis in the MCP-fed nude mice into which human colon carcinoma cells (LSLiM6) were implanted than in the control mice. Metastases to lymph nodes and the liver were present in 100% and 66% of control mice versus 25% and 0% of mice fed with MCP.

Pectin consists of "smooth" and "hairy" regions. The smooth region is composed of partially esterified galacturonic acid residues, whereas hairy regions contain galacturonic acid residues with irregularly inserted rhamnose residues with side chains composed of neutral sugars such as arabinose, galactose, glucose, mannose, and xylose. The modification of CP to MCP by pH involves degradation of the main galacturonic acid chain by β elimination (high pH) followed by partial degradation of the natural carbohydrates (low pH), resulting in simpler carbohydrate chains of basically the same sugar composition as the unmodified CP. Composition analysis of CP and MCP showed that MCP is richer in galactose, rhamnose, and xylose (data not shown). MCP effectively binds to recombinant galectin-3 and inhibits galectin-3-mediated functions, such as homotypic tumor cell aggregation, binding of tumor endothelial cells, anchorage-independent growth, and binding to the laminin ligand by blocking its carbohydrate-binding domain *(18–20).* Our results show that MCP also acts as an angiogenesis inhibitor. In an *in vitro* assay, HUVECs migrated and differentiated into capillary-like structures, and MCP prevented this migration and capillary tube formation, either by binding to the galectin-3 present in the matrix and/or on the endothelial cells or interfering with its binding to the receptor. *In vivo* this leads to a marked reduction in the density of tumor-associated blood vessels. Similarly, MCP specifically inhibited the binding of the galectin-3-expressing breast cancer cell line MDA-MB-435 to endothelial cells, explaining in part its inhibition of invasion and metastasis. Recent findings demonstrate that hematogenous cancer metastases originate from intravascular growth of cancer cells attached to the endothelium rather than from extravasated ones *(34).* This

suggests a key role of tumor–endothelial cell interactions in cancer metastasis. Here we have shown that MCP inhibited galectin-3-induced and bFGF-induced chemotactic migration, and others have reported that CP inhibits the binding of bFGF to its receptor FGFR in the presence of heparin, also a complex carbohydrate *(35)*.

In summary, our results demonstrate that MCP inhibits *in vitro* and *in vivo* carbohydrate-mediated angiogenesis by blocking the association of galectin-3 to its receptors. These data stress the importance of dietary carbohydrate compounds as cancer-preventive and/or -therapeutic agents. The complex nature of carbohydrate specificities will require the development of new antagonists for the recognition of angiogenic factors and glycoconjugate receptors.

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NOTES

Supported in part by Department of Defense grant BC991100; Public Health Service grants RO1CA46120 and RO1CA69480 from the National Cancer Institute, National Institutes of Health, Department of Health and Human Services; and by grant DE-FG09-93ER-20097 from the Center for Plant and Microbial Complex Carbohydrates (funded by the Department of Energy).

Manuscript received January 16, 2002; revised August 23, 2002; accepted October 1, 2002.